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Metabolic Interrelations Between Intrinsic Factor and Vit. B_{12}.* III. B_{12} Absorption at Varied Intrinsic Factor Doses. (21476)

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It has been observed in hematological studies that an increased intake of vit. B_{12} with a constant dose of intrinsic factor increases the absorption of vit. B_{12}, and, vice versa, an increased intake of the intrinsic factor with a constant dose of vit. B_{12} also enhances the absorption of vit. B_{12}(1,2). These inferences were drawn from reticulocyte and red cell responses of patients with pernicious anemia in relapse and the information obtained was limited because of the inherent difficulties of these hematological assays. With the availability of the Co^{60}-tagged vit. B_{12}(3) and the development of new technics(4-7) for the study of the intestinal absorption of vit. B_{12} it became possible to re-investigate the interrelations between oral doses of B_{12} and of intrinsic factor also on patients with pernicious anemia in remission. In previous work, we used the surface scintillation method of measuring the uptake of radioactive vit. B_{12} by the liver(6,7) to determine its intestinal absorption(8). These studies indicated that in normals the intestinal absorption of vit. B_{12} rises with increase of the dose, as was shown earlier by hematological studies(1,2). However, the efficiency of the absorption of vit. B_{12} in the intestine rapidly decreases with increase of the dose, so that the increment in the absolute amount of B_{12} absorbed, even with a large increase of the ingested dose from 0.5 to 50 μg increases the amount absorbed from about 0.4 to about 1.5 μg. The same principle of regressing efficiency of absorption with increase of the dose of B_{12} ingested also holds in patients with pernicious anemia who receive vit. B_{12} with preparations of intrinsic factor orally(8).

The effect of an increased dose of intrinsic factor with constant B_{12} intake upon the intestinal absorption of vit. B_{12} was investigated with the isotope method only casually. In 2 cases reported so far, the absorption of B_{12} was increased with an increase in the dose of intrinsic factor(4,5). On the other hand, Rosenblum *et al.* showed on normal(9) and gastrectomized(10) rats that the addition of intrinsic factor to radioactive Co^{60}-B_{12}, did not

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TABLE I. Uptake of Radioactivity by the Liver following Oral Administration of Co^{60}-B_{12} Alone and Together with Various Doses of Intrinsic Factor Containing Preparations.*

Case	Diagnosis	Material admin. by mouth†	Hepatic uptake of radioactivity above background‡	
			c. p. m.	c. p. m./1 μc Co^{60} ingested
W. B. ♂ 68	Pernicious anemia in remission	0.5 μg Co^{60}-B_{12} (0.37 μc Co^{60}) alone	0	0
		Same + 12 mg fraction C-IV‖	0	0
		24	28	76
		37	62	168
J. d'A. ♀ 67	Total gastrectomy 1 yr ago for carcinoma of stomach	0.5 μg Co^{60}-B_{12} (0.37 μc Co^{60}) alone	0	0
		Same + 31 mg IF-0-5333	49	133
		62	149	403
A. C. ♀ 72	Normal control	1 μg Co^{60}-B_{12} (0.21 μc Co^{60}) alone	90	442
		Same + 58 mg IF-0-4635	48	235
		116	51	250

* Data listed in this table not contained in Fig. 1.

† Materials listed have been described in text and in the legend to Fig. 1.

‡ Each figure represents avg value calculated from increments in radioactivity uptake over 3 skin projections of liver on 6th and 7th day following oral administration of single dose of radioactive vit. B_{12} alone or together with intrinsic factor containing preparations. Figures corrected for spontaneous decay of Co^{60}.

increase the intestinal absorption of vit. B_{12} in these animals. The present study is intended to add more detailed and systematic data to this problem.

Method. The measurements of the intestinal absorption of vit. B_{12} and the activity of the intrinsic factor preparations were done by the hepatic uptake method(6,7). This is based on determination of the increment in the radioactivity counts over the liver following the oral administration of the intrinsic factor preparation together with 1-2 μg of radioactive vit. B_{12} of specific activity 212 $\mu c/mg$, *i.e.* containing 0.21–0.42 microcuries Co^{60}, or with 0.5 μg of radioactive B_{12} of high specific activity 770 $\mu c/mg$, *i.e.* containing 0.385 μc Co^{60}. The counts were taken with the scintillation counter without collimator and provided with a NaI-Thallium crystal 1″ in diameter and ½″ in depth at 1000 volts, in direct contact with the skin over the anterior, antero-lateral and mid-lateral projections of the liver on the 6th and 7th day after ingestion of radioactive B_{12}. We used a scaler with preset time and precise regulation of the high voltage and sensitivity. Four to 6 projections from other areas of the lower, mid and left abdomen are used as controls to rule out the occasional interfering retention of radioactive material in the intestine. The uptake of radioactivity over the liver in counts per minute is averaged from all three liver projections on both days of assay, calculated above background, and corrected for the efficiency of scaler and decay of Co^{60} with the use of dried Co^{60}-B_{12} standard; the final figures are then calculated per 1 μc Co^{60}.

In normal subjects the administration of radioactive vit. B_{12} causes the uptake of a detectable amount of radioactivity by the liver which acts as a target organ for B_{12} absorbed; this count exceeds significantly that over the rest of the abdomen(6,7). In pernicious anemia, both in relapse and remission, no uptake is observed, so that the counts over the other abdominal organs are similar or even higher than over the liver. However, when an active intrinsic factor material such as normal gastric juice or a potent intrinsic factor concentrate from animal stomach is added, a positive uptake of radioactivity over the liver follows and indicates the promoting effect of material containing intrinsic factor on the absorption of B_{12}. We used this method on patients with pernicious anemia in remission, and demonstrated its equivalence(11,12) to the classical method of hematopoietic responses which involves patients with pernicious anemia in relapse.

Results. Effect of varying doses of intrinsic factor concentrate from hog stomach, at same

Hepatic uptake of radioactivity in 6 cases of pernicious anemia in remission following oral administration of 0.5-2 μg Co^{60}-B_{12} with varying doses of intrinsic factor containing materials.

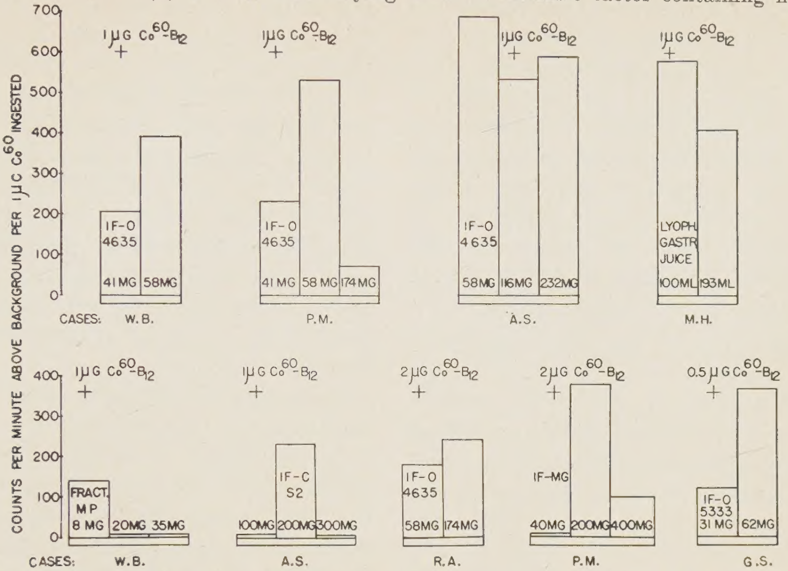

FIG. 1. Measurements of hepatic uptake performed in 6 cases of pernicious anemia, first following oral administration of Co^{60}-B_{12} alone, without addition of intrinsic factor. This resulted in all instances in no hepatic uptake of radioactivity, indicative of an intestinal block to absorption of vit. B_{12}, typical of pernicious anemia. Addition of intrinsic factor to the same oral dose of radioactive vit. B_{12} promoted intestinal absorption of some part of Co^{60}-B_{12} ingested, resulting in a positive hepatic uptake of radioactive vit. B_{12}. Each figure represents avg value from increments in radioactivity uptake over 3 skin projections of liver on 6th and 7th days following oral administration of radioactive Co^{60}-B_{12}. Counts taken at 1000 volts in close contact with skin, and calculated above background per 1 μc of Co^{60} ingested. Specific activity of Co^{60}-B_{12} was 0.213 μc/μg at a dose of 1 or 2 μg Co^{60}-B_{12}, and 0.755 μc/μg at 0.5 μg Co^{60}-B_{12}. Following preparations containing intrinsic factor were used:

Intrinsic factor concentrate #4635 Organon, Inc., listed as IF-0-4635;
#5333 IF-0-5333;
''G'' Merck Co. IF-MG;
P-160 Cudahy Lab. IF-C-S2;
Lyophilized mucoprotein fraction from human gastric juice, listed as Fract. MP;
" pooled human gastric juice, listed as Lyoph. g. j.

dose of vit. B_{12}. Six patients with pernicious anemia in remission, one patient with total gastrectomy, and one normal control individual, received a tracer dose of 0.5, 1 or 2 μg radioactive B_{12}, containing 0.385, 0.21 or 0.42 μc Co^{60}-B_{12} respectively. After the hepatic uptake of radioactivity was determined on the 6th and 7th days, other similar doses of Co^{60}-B_{12} were given by mouth at intervals of 1-2 weeks, to which were added varying doses of potent intrinsic factor concentrates from the hog stomach. In cases W.B., P.M., A.S., R.A. and A.C., preparation IF-0-4635 was used in doses ranging from 41 to 232 mg[†]; in case GS. and J.d'A.—preparation IF-0-5333[†] in a dose of 31 and 62 mg; in case P.M.—preparation IF-MG[‡] in a dose of 40, 200 and 400 mg; and in case A.G.—preparation IF-C-S2[§] in doses of 100, 200 and 300 mg. The data calculated per 1 μc Co^{60} ingested are shown in Fig. 1, and in Table I.

In patients with pernicious anemia we obtained highest hepatic uptake figures indicative of high intestinal absorption of vit. B_{12}, with a dose of 58 mg of the intrinsic factor preparation IF-O-4635 (cases W.B., P.M., A.S.), and the dose of 1 μg Co^{60}-B_{12}. This dose of intrinsic factor is equivalent in potency to that contained in one oral USP Anti-Anemia Unit of Bifacton, Organon Inc. When the dose of intrinsic factor was increased to 116, 174 or 232 mg, or decreased to 41 mg, the uptake of radioactive B_{12} by the liver decreased, indicative of decreased intestinal absorption of vit. B_{12} under these conditions, except for case R.A., which will be discussed below, and a control subject where it didn't change.

[†] Kindly supplied by Dr. Kenneth Thompson from Organon Inc., Orange, N. J.

[‡] Kindly supplied by Drs. Nathaniel Ritter and Charles Rosenblum from Merck Co., Inc., Rahway, N. J.

[§] Kindly supplied by Mr. H. J. Anderson, from Cudahy Laboratories, Chicago, Ill.

On the other hand, 31 mg of another intrinsic factor concentrate (IF-0-5333), and given with the standard dose of 0.5 μg Co^{60}-B_{12} in two cases (G.S. and J.d'A.), produced only one-third of the hepatic uptake of radioactivity, as compared to that when the dose of the IF was doubled to 62 mg with the same dose of B_{12}.

Using still another intrinsic factor preparation (IF-C-S2) we found that it was absolutely inactive at a dose of 100 mg, fairly active at the dose of 200 mg and again entirely inactive at the dose of 300 mg when added to a constant dose of 1.0 μg Co^{60}-B_{12} containing 0.21 μc of Co^{60}. Finally, intrinsic factor concentrate (IF-MG) has shown optimal activity in a patient with pernicious anemia in relapse (P.M.) at the level of 200 mg, lower activity at the level of 400 mg, and no activity at 40 mg dose.

Effect of varying doses of intrinsic factor materials from human gastric juice in patients with pernicious anemia, at same dose of vit. B_{12}. In 2 patients with pernicious anemia we used intrinsic factor materials of human origin: a) pooled and lyophilized human gastric juice, collected under fasting conditions from various patients and neutralized and dialyzed before lyophilization; b) one of the mucoprotein fractions (MP) processed in our laboratory from the human gastric juice; c) one of the chromatographically separated fractions (C-IV) from the pooled human gastric juice.‖ The results of these studies (Fig. 1 and Table I) were as follows:

With the doubling of the dose of lyophilized gastric juice (from 100 to 193 ml) the uptake of radioactive Co^{60}-B_{12} decreased in one case studied (M.H., Fig. 1). Similarly in case W.B., the administration of 8 mg of mucoprotein fraction from human gastric juice with 1 μg Co^{60}-B_{12} resulted in a slight uptake of radioactivity by the liver, while 20 and 35 mg resulted in no radioactive hepatic uptake at all. On the other hand, the chromatographically separated fraction C-IV, was entirely inactive at the dose of 12 mg, slightly active at the dose of 24 mg and moderately active at a dose of 36 mg.

Discussion. The results listed in Fig. 1 and Table I, confirm previous inferences drawn from hematological method which indicated the increase in the intestinal absorption of vit. B_{12} in pernicious anemia with increased intake of intrinsic factor and unchanged dose of vit. B_{12}(1,2). This is shown by rise of the hepatic uptake of Co^{60}-B_{12} in cases W.P. and P.M. (Fig. 1) on increase of the dose of IF-0-4635 from 41 to 58 mg, in case G.S. (Fig. 1) and J. d'A. (Table I) on that of IF-0-5333 from 31 to 62 mg, in cases P.M. and A.G. (Fig. 1), on that of IF-MG or IF-C-S2 from 40 respectively 100 mg to 200 mg, and in case W.B. (Table I), on that of C-IV from 12 to 24 and 36 mg. However, the increase in the intestinal B_{12} absorption apparently goes only to a certain optimal point beyond which the addition of an excess of intrinsic factor becomes devoid of any enhancing effect or may even paradoxically decrease the absorption of B_{12} in the intestine. This is shown by data obtained in cases P.M. and A.S. (Fig. 1), where the further increase of the IF-0-4635 from 58 to 116-232 mg, or IF-C-S2 or IF-MG from 200 to 300 or 400 mg respectively, caused the drop in hepatic counts, or in a normal control patient (Table I) where the addition of intrinsic factor concentrate markedly decreased the intestinal absorption of Co^{60}-B_{12}. Similar inferences can be drawn from data in cases M.H. and W.B. (Fig. 1), in whom similar decrease in intestinal absorption of vit. B_{12} was obtained on excessive increase of the dose of intrinsic factor containing material from human sources. What the optimal level of intrinsic factor is, obviously depends on the potency of the preparation, but also on the dose of vit. B_{12} used with it. This is exemplified by the rise in the uptake of B_{12} on increase of the dose of intrinsic factor from 58 to 174 mg when given with 2 μg Co^{60}-B_{12} (case R.A., Fig. 1), and decrease in the hepatic uptake under similar conditions, when given with 1 μg Co^{60}-B_{12} (case P.M., upper row, Fig. 1).

‖ Kindly supplied by Dr. R. Caputto from Dr. Stewart Wolf's Research Laboratory in Oklahoma University Medical School.

The underlying mechanism of this effect is not clear but it may depend on some binding of vit. B_{12} in the lumen of the intestine by the excessive amounts of vit. B_{12} binding substances contained in the heterogenous mixtures of the intrinsic factor concentrate or in gastric juice. This seems plausible because of great affinity for vit. B_{12} exhibited by one of the mucoproteins contained in the intrinsic factor concentrate from hog stomach ("red cobalamin-protein")(13), as well as presence of other proteins in the human gastric juice which have high capacity for binding vit. B_{12} (14). Another possibility is that there may exist an optimal concentration of intrinsic factor in the intestine which is necessary for the optimal absorption of a certain amount of vit. B_{12}, so that any deviation to either side of this optimum might inhibit the intestinal absorption of this vitamin. This factor is of great practical importance in oral treatment of pernicious anemia(15). It adds also to the complexity of the assay of the potency of intrinsic factor preparations. If this factor is not taken into consideration and the assay is not performed at the optimal ratio, the resultant inferences may be quite erroneous (see cases P.M. or A.S., Fig. 1). It appears that merely to determine the activity of intrinsic factor preparation tested at one dose level and with only one dose of B_{12} is entirely inadequate for the evaluation of its potency.

Conclusions. Increase in oral dose of intrinsic factor preparations from hog stomach or active gastric materials from humans, with a constant oral dose of vit. B_{12}, up to a certain point, increases the intestinal absorption of vit. B_{12} as measured by the hepatic uptake of Co^{60}-B_{12}. However, further increase in the amount of intrinsic factor may be devoid of any enhancing effect or even result in decreased absorption of B_{12} in the intestine. The latter might be due to an excessive binding of B_{12} to the non-absorbed fraction of the intrinsic factor preparation, having high binding capacity for vit. B_{12}.

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Temperature Shock in Human Spermatozoa. (21477)

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Two types of death can result during the exposure of cells to extremely low temperatures. One occurs during rapid cooling above the freezing temperature and the other is associated with ice formation. Since it is possible that both are operative during the freezing of cells, it seems essential to separate the two before evaluating the lethal processes involved.

Cold shock(1), temperature shock(2), thermal shock(3), and constitutional cold death (4) are terms used in the literature to describe the harmful consequences of rapidly cooling cells above 0°C. It is believed that the majority of mammalian cells are, to some extent, subject to this phenomenon and that among isolated cells and tissues its deleterious effects are best known with spermatozoa(5). However, the results of some studies on freezing human spermatozoa(6,7) prompted the author to suggest that these cells were not susceptible to this type of death. The purpose of this communication is to present findings which support this suggestion.

Methods. It was decided to utilize technics suggested in the literature(1,8,9) and improvise others in order to ascertain whether or not human spermatozoa were subject to temperature shock at different cooling rates. Semen, considered normal by the usual seminologic tests(10), was collected on 2 occasions from each of 9 donors. One ejaculate from each donor was treated with glycerol after the method of Sherman and Bunge(11) while the other was not. Semen in the presence and absence of glycerol was used in the subsequent experimentation. This permitted a possibly interesting comparison since glycerol is used as a protective substance in the preservation of human spermatozoa at low temperatures. In each experiment a sample of semen was analyzed immediately before and 5 minutes after its exposure to cold. Viability was measured by the eosin-nigrosin staining technic of Blom(12), while the percent, speed, and duration of motility was obtained essentially by procedures suggested by Farris(10). The type of motility was also observed. Experiments performed are listed in Table I. An additional experiment was run to observe possible effects of rapidly rewarming semen above the freezing temperature. The procedure was the same as in Exp. 3 except that temperature of staining solution was 30°C while temperature of semen was varied.

TABLE I. Tests for Temperature Shock in Human Spermatozoa.

Exp. No.	
1	Cold shock test(1): 1 cc of semen (25°C) placed in 10 × 75 mm bacteriological test tube. Tube corked and placed in water bath (0°C) for 10 min.
2	Same as 1 except that bath (salt water) was at –4°C and exposure time 3 min.
3	Hancock test(9): one drop of semen (30°C) mixed on 3″ × 1″ glass microscope slide with eosin-nigrosin staining solution at various temperature levels between 0 and 30°C. Specifically, one drop of semen at 30°C was mixed with one drop of staining solution at 0, 3, 6, 9, 12, 15, 18, 21, 24, 27, or 30°C.
4	One drop of semen (25°C) placed on 3″ × 1″ glass microscope slide, slide then placed on melting ice for 5 min.
5	Same as 4 except that ice was at –15°C and slide kept on it until ice first seen forming at edge of semen drop.
6	Same as 5 except that dry ice (–80°C) was used as refrigerant.

Results. There was no change in either the viability or motility of spermatozoa in any of the semen samples exposed to the experimental conditions described. In Exp. 5 and 6, when semen was observed immediately after the cooling period instead of following the usual 5 minutes wait, there were no motile spermatozoa. However, original motility returned after less than 2 minutes at room temperature.

Discussion. Smith and Polge(13) stressed the importance of temperature shock in the lethal aspect of freezing spermatozoa. Indeed, definite gradual rates of cooling above the freezing temperature are recommended in the technics for preserving bull spermatozoa at low temperatures(14). On the basis of the findings reported here, however, no such precautions are needed with human spermatozoa.

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Mammalian semen, other than that of the human, requires the presence of a protective substance such as glycerol if any of its spermatozoa are to survive exposure to low temperatures(13). Such spermatozoa, those of the bull for example, are also subject to temperature shock. Glycerol protects these spermatozoa during freezing but its action is generally considered to be associated with ice formation (5). The results of Lovelock(3) suggest a common lethal factor which operates above and below the freezing temperature and that egg yolk (lecithin) as well as glycerol minimize its effects. Is it not possible then that glycerol may also act to lessen sensitivity to temperature shock? Egg yolk(15) probably its lecithin portion(16) has been shown to protect these cells against temperature shock. Bull semen diluents employed in preservation at low temperatures contain both egg yolk and glycerol. Therefore, it seems that the possible protective role of glycerol in temperature shock remains to be evaluated.

Summary. 1. Human semen in the presence and absence of glycerol was cooled at various rapid rates above the freezing temperature in order to ascertain whether or not the spermatozoa exhibited temperature shock. It was found that these cells, unlike the majority of other mammalian cells, are not susceptible to this phenomenon. It is indicated, therefore, that the death of spermatozoa, which occurs when human semen is exposed to extremely low temperatures, is associated with ice formation. 2. Spermatozoa in human semen in the presence and absence of glycerol showed no deleterious effects when they were rapidly rewarmed above the freezing temperature. Therefore, if death occurs during the thawing of frozen human semen, then most likely it too is associated with ice formation. 3. The possible role of glycerol in temperature shock is discussed.

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Infection of Monkey Kidney Tissue Cultures with Virus-Like Agents.* (21478)

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The wide current interest in tissue cultures for the study of virus-host cell relationships and applied problems of virus diseases has emphasized the need of employing cells and tissues free of microbial agents. The hazard of introducing viruses into tissue cultures with contaminated media has been stressed recently (1). Of equal, if not greater importance, is the problem of the presence of viruses in tissue cultures as a result of unrecognized infection of cells and tissues employed as primary explants. Detection of such viral infections, in certain instances, may be complicated by the fact that no readily recognized changes are manifested. On the other hand, some viruses under the same conditions may subsequently interfere markedly with cellular growth as evidenced by inhibition of acid production or cytological changes.

In our attempts, in 1953, to adapt mouse-adapted Hawaii dengue virus(2) to roller tube cultures of rhesus monkey kidney, an unidentified agent was encountered which induced cytopathogenic changes in cultures of monkey kidney and cancer HeLa epithelial cells. The agent was filterable and could not be cultured in various non-living media. Subsequently, 3 additional agents with identical cytopathogenic characteristics were passed from uninoculated monkey kidney cultures prepared for poliomyelitis studies, to HeLa cell cultures. Enders and Peebles(3) have recently reported recovery of an agent from an uninoculated monkey kidney culture which appears to have the same cytopathogenic characteristics in monkey renal cultures as our agents have. However, other than a description of its cytopathic effect in monkey kidney cultures, no other data was given concerning their agent. In view of the wide use of monkey kidney cultures in virus studies it seems of importance to report our observations with these agents. These include their cytopathic effect in rhesus monkey renal tissue, in HeLa cell and certain other tissue cultures, the manner of their recovery and passage in tissue culture and other characteristics. In addition, evidence is presented that kidneys of apparently healthy monkeys are the source of these agents in monkey kidney tissue cultures and not the medium constituents.

Materials and methods. Tissue culture technics. A. *Roller tube cultures.* Technics of Robbins, Weller and Enders(4) were essentially followed for preparation of roller tube cultures. In addition to rhesus monkey kidney, cultures were prepared from monkey testes, human embryonic kidney, human embryonic skin and muscle† and mouse infant kidney. In early studies the culture medium consisted of Hanks-Simms solution (70%), beef embryo extract (10%) and horse serum (20%). Penicillin and streptomycin were added for final concentration of 100 units and 100 μg respectively. Later, bovine amniotic fluid was used in place of Hanks-Simms solution(5). Soybean trypsin inhibitor, in final concentration of .05 mg, was incorporated in medium for all cultures. One ml of medium was added to each culture tube containing 10 to 15 fragments of tissue. Culture fluids were changed every 3 to 4 days. At time of inoculation of agents, the medium was modified to contain 85% Hanks-Simms or bovine amniotic fluid, 10% beef embryo extract and 5% horse serum. B. *Stationary cultures* of monkey kidney were prepared by trypsinization technic of Youngner(6) with bovine amniotic fluid containing 5% beef embryo extract and 10% horse serum. Following standardization of cell suspensions(7) 0.5 ml containing about 500,000 cells was added to culture tubes.

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† We are grateful to Dr. W. P. Mavralis, Illinois Central Hospital, for making human embryo available for tissue culture.

When good cell growth occurred (7 to 9 days), the medium was changed to 95% bovine amniotic fluid and 5% horse serum. C. *HeLa cell* strain of cancer epithelial cells, kindly supplied by Dr. J. T. Syverton of the University of Minnesota, was cultured as described by Scherer, Syverton, and Gey(8) and Syverton (9). Culture tubes were seeded with approximately 40,000 cells in 1 ml, and in 4 to 5 days a layer of growth was obtained containing 90,000 to 160,000 cells. The nutrient fluid was then replaced with maintenance solution containing 10% chicken serum(8,10). With prolonged incubation of cultures in this fluid, degenerative changes were noted (granulation, rounding of cells). When this occurred the maintenance medium was replaced with nutrient fluid which restored normal morphology in 1 to 2 days. Accordingly, in experiments involving long incubation periods, nutrient fluid was substituted for maintenance medium and left on the cells for brief intervals. *Preparation and staining of cells for histologic study.* Examinations of stained tissue culture cells from infected monkey kidney and HeLa cell cultures were made. Each monkey kidney culture was prepared by placing a kidney fragment on cover slip with chicken plasma which was then clotted with chick embryo extract. The cover slip was then placed in a roller tube containing 10 to 15 fragments. To obtain HeLa cells for staining, a cover slip was placed in a Pritchard tube which was then inoculated with cell suspension and sealed with rubber stopper. Following infection of the cells the cover slips were removed, fixed in Zenker's solution containing 10% formalin, dehydrated and stained with hematoxylin and eosin.

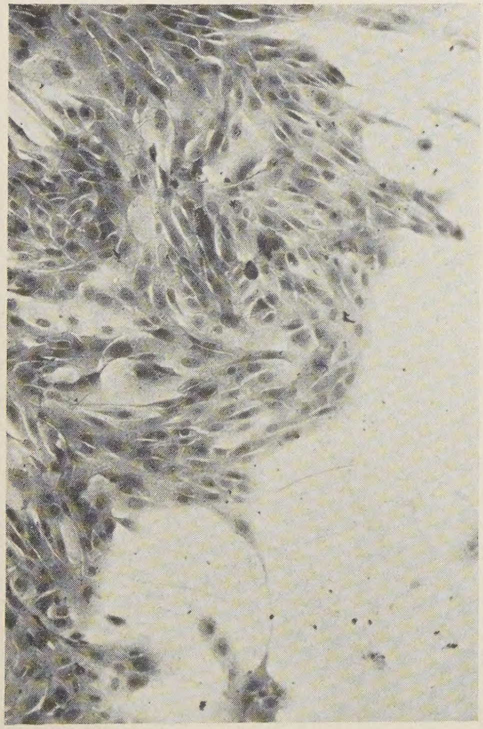
FIG. 1. Outgrowth of epithelial cells of uninoculated monkey kidney tissue culture 15 days after preparation (×90).

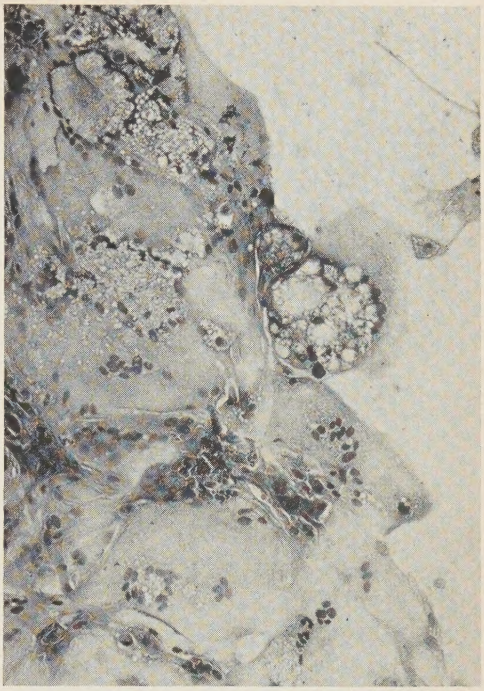
FIG. 2. Infection of epithelial cells of a monkey kidney culture 5 days after inoculation with agent MK-D showing vacuolated syncytial structures (×90).

Experimental. Cytopathic characteristics of agents in tissue cultures.‡§ A. *Monkey kidney epithelial cells.* Degeneration of epithelial cells of monkey kidney cultures containing

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‡ We are indebted to Dr. Robert W. Wissler, Department of Pathology, for aid in interpreting cytological changes.

§ Cytopathic effects of agents are presented prior to manner of recovery to facilitate description of their recognition and passage in tissue culture.

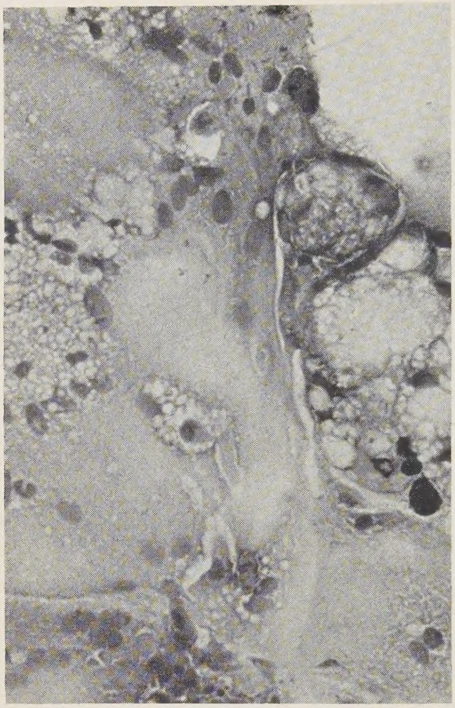

FIG. 3. Same as Fig. 2 at a higher magnification (×207).

these agents usually could be seen under low power magnification (100X) after 5 to 8 days. Cellular changes usually appeared first at or near the periphery of outgrowth of epithelial cells. There were areas in which individual cells were no longer visible but appeared to be made up of degenerated and perhaps "fused" cells, dull in appearance and with "ill defined" cell boundaries. Clusters of nuclei could be distinguished. The multiple nuclei, grouped together, against the dull background suggested large fused syncytial structures. There appeared, at the same time, variable sized vacuoles which in some instances were more prominent than the syncytial structures. With further incubation the number of "fused" and vacuolated areas increased until few if any patches of normal cells remained. Degeneration was slow, 1 to 2 weeks were required after onset for practically complete degeneration. Examination of stained preparations of infected monkey kidney cells confirmed these observations with unstained cells (Fig. 1, 2, 3). These reactions in monkey kidney cultures appear to be similar to those obtained by Enders and Peebles(3) with their monkey renal agent which they encountered during studies with agents from measles patients. The reactions also resemble those of their measles agents in human as well as monkey kidney cultures. The measles agents, however, differed in that they caused marked intranuclear changes.

B. *HeLa epithelial cells.* Following a similar incubation period degenerated areas containing cellular substances and clusters of nuclei were much more sharply defined than in monkey kidney cultures and could be seen macroscopically as lytic-like areas (Fig. 4, 5). Of much greater contrast to reactions in monkey kidney cultures was the absence of vacuolation. Small refractory masses which may have been vacuoles were seen at the time of marked degeneration, rare and difficult to define. Degeneration seemed to consist mainly of a gradual increase in size and number of lytic-like areas and their subsequent coalescence. At the same time multinucleated cellular masses representing either giant cells or syncytial structures became more prominent. It should be noted that, on occasions, small

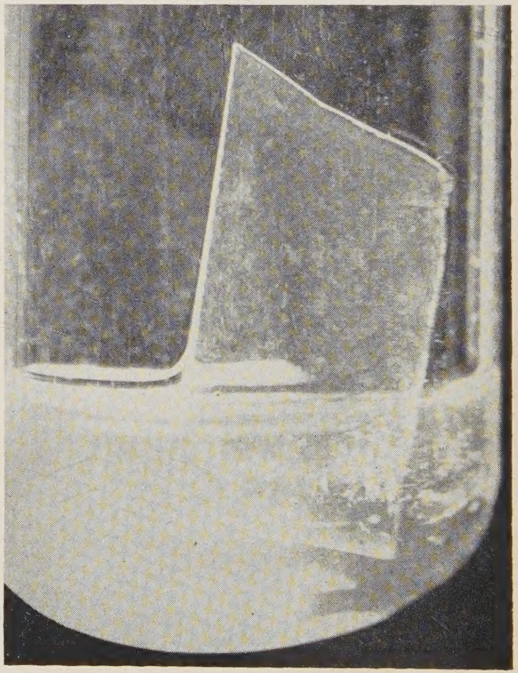

FIG. 4. Macroscopic appearance of normal film of unstained HeLa cells grown on a portion of a cover slip in a Pritchard tube.

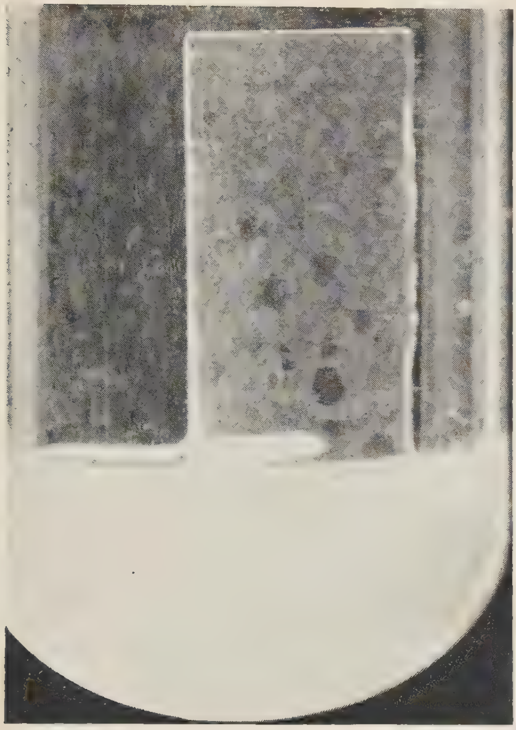

FIG. 5. Film of unstained HeLa cells on cover slip in Pritchard tube showing macroscopic appearance of well defined lytic-like areas 8 days after inoculation with agent MK-D.

lytic-like areas with giant-cell forms were observed in uninoculated HeLa cell cultures. However, they were never as marked as in infected cultures nor did they increase in size. Moreover, blind passage experiments with such cultures on two different occasions not only failed to enhance the reaction, but did not give rise to its consistent occurrence. Staining of infected HeLa cell cultures did not reveal any additional changes (Fig. 6,7,8).

Cytopathogenesis in other types of tissue cultures. The cytopathogenic effect of one agent in its 8th monkey kidney passage was tested in rhesus monkey testicular cultures, human embryonic kidney, human embryonic skin and muscle, and infant mouse kidney. In addition, pooled fluid from uninoculated monkey kidney cultures, from which one agent was recovered in HeLa cell cultures, was passed to human embryonic kidney and human embryonic skin and muscle cultures. Cytopathic effects were obtained in all instances except in mouse kidney cultures. Epithelial cells of human embryonic kidney showed degeneration as in monkey kidney cultures. However, degeneration of fibroblastic growth in monkey testicular and human skin and muscle cultures differed in that no syncytial structures nor vacuolation were observed, but the fibroblasts appeared to undergo a granular fragmentation.[‖]

Recovery of agent MK-D during attempts to adapt dengue virus to monkey kidney cultures. Following inoculation of the mouse-adapted strain of Hawaii dengue virus (137th mouse passage) into monkey kidney cultures, no cytopathic effects were observed for 16 days. Nevertheless, fluid harvested on 12th day from 3 cultures was pooled and passed into fresh 9-day-old monkey kidney cultures. With this second passage cellular changes, as described, occurred in all 5 inoculated cultures in 8 to 20 days. But of 3 uninoculated control cultures held for 33 days, one revealed

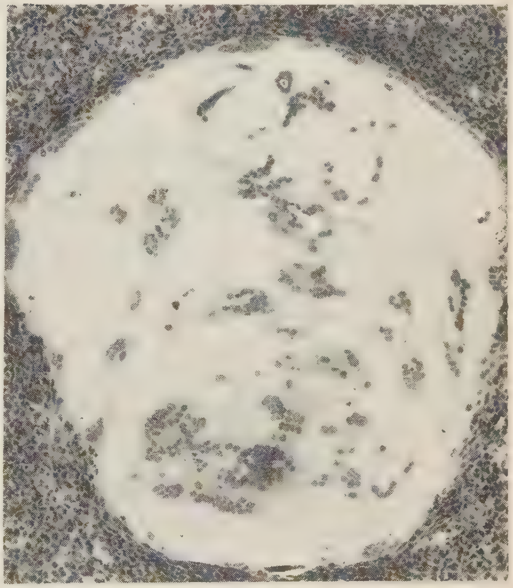

FIG. 6. Stained film of HeLa cells showing lytic-like area containing giant cells or syncytial structures 8 days after inoculation with agent MK-D. Note absence of vacuolation (×44).

[‖] Dr. J. S. Youngner, Virus Research Laboratory, University of Pittsburgh, has kindly given us permission to state that he has also observed granular fragmentation in monkey testicular cultures with similar agents he recovered from uninoculated monkey kidney cultures.

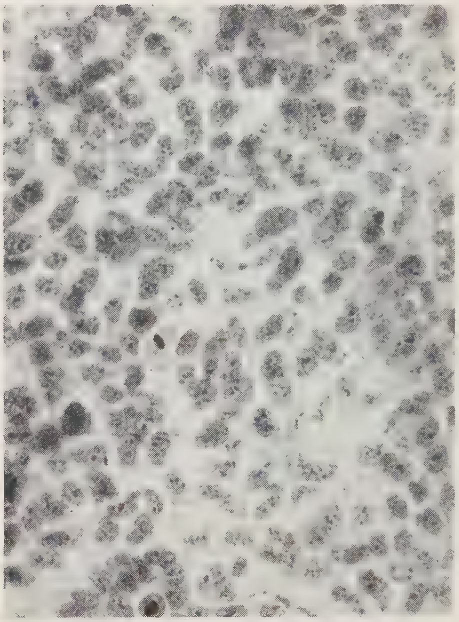

FIG. 7. High magnification of 14-day-old culture of stained normal HeLa cells (×207).

identical degeneration in 19 days. This culture, unfortunately, was discarded. With third passage 3 cultures gave reactions in 10 days, and by 4th passage the incubation period decreased to 6 to 8 days. Fifteen serial passages have now been made with this agent in monkey kidney cultures and starting with 8th monkey kidney passage, 9 serial passages in HeLa cell cultures. With the first tissue culture passage of dengue virus, infant mouse inoculations were made with fluids harvested at 4-day intervals and continued through 7th tissue culture passage. Fluids from 1st and 2nd tissue culture passages produced signs of disease compatible with dengue, but not fluids from subsequent passages. Further studies, including challenge experiments in mice with mouse dengue virus and neutralization tests with dengue serum conclusively demonstrated that the agent established in tissue cultures was not dengue.

Recovery of agents MK1, MK3, and MK4 from uninoculated monkey kidney cultures. Shortly after encountering agent MK-D in attempts to adapt dengue virus to monkey kidney cultures, syncytial masses and vacuolation were again observed in an uninoculated roller tube culture 12 days after its preparation. This culture and 16 others prepared from the same kidney were set aside for further study. By 15th day degeneration was marked in this culture and had appeared in 3 other cultures; and by 34th day in 7 of the 16 cultures. Fluids were harvested on 14th and 15th day from cultures showing degeneration, pooled and 0.2 ml inoculated into HeLa cell cultures. Lytic-like areas were present in 10 days. Fluids from these cultures were then harvested, pooled and 0.1 ml passed to fresh HeLa cultures. Similar degeneration with this second passage occurred in 6 days. Subsequent passages in HeLa cells were made with 0.1 to 0.2 ml of pooled fluids harvested at 1- to 2-day intervals for 4 to 6 days following definite signs of degeneration. The fluids were stored in a CO_2 cabinet for varying periods between passages. This agent, designated MK1 has now been passaged serially 10 times in HeLa cell cultures. *Agents MK*3 *and MK*4 were recovered similarly from uninoculated cultures prepared from kidneys of different rhesus monkeys. Thus, MK3 was recovered from a series of uninoculated cultures in which 8 of 72 roller tubes revealed moderate to strong

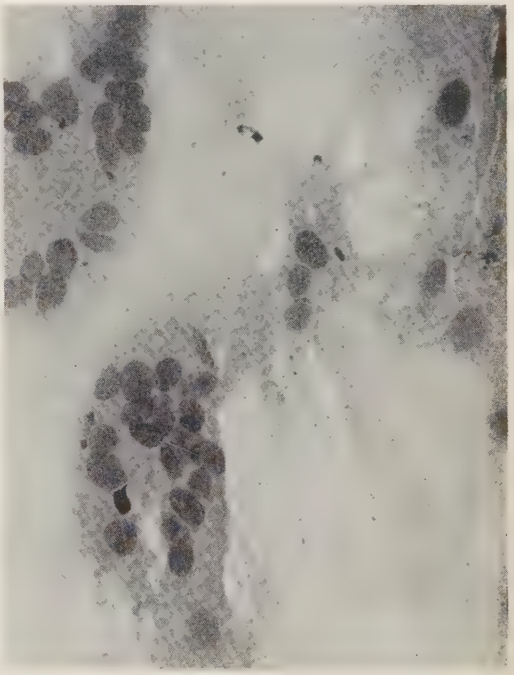

FIG. 8. High magnification of giant cells or syncytial structures in HeLa cell culture infected with agent MK-D (×207).

TABLE I. Frequency of Syncytial Structures and Vacuolation in Uninoculated Monkey Kidney Cultures Prepared from Kidneys of 12 Rhesus Monkeys.

				Degeneration				
Kidney series*	Date prepared	No. kidneys	Days of observation	No.†	Degree	Time of appearance (days)	Isolation of agent	Neutralization by homologous serum
21	11/13/53	1	34	7/16	++ to +++	12–24	+	N.D.§
22	12/ 5	1	23	0/25	±‡		—	+
23	12/19	1	55	0/13	"		N.D.	—
24	1/ 6/54	1	20	11/18	++ to +++	12–20	+	+
25	1/13	1	23	2/ 7	+++	15–21	N.D.	+
32	6/25	2	28	0/15	±‡		"	N.D.
39	8/11	4	34	13/41	++ to +++	19–24	+	"
42	8/ 2	4	28	3/14	*Idem*	20–23	N.D.	Monkey 1: + " 2: —
43	9/13	4	34	1/10	++	31	"	N.D.

* Kidney series 21 through 25, roller tube cultures; 32 through 43, stationary cultures with trypsinized cell suspension.

† Numerator = No. of cultures showing reaction; denominator = No. of cultures.

‡ Slight vacuolation in practically all cultures.

§ N.D. = Not done.

degeneration 12 days after preparation and MK4 from 11 of 41 stationary cultures with degeneration 20 days after preparation with trypsinized cell suspension. These 2 agents have now been serially passed 10 and 6 times respectively in HeLa cultures. MK3 following 10 HeLa cell passages caused vacuolation and formation of syncytial masses in monkey kidney cultures. Same results were obtained with MK4 after 6 HeLa cell passages. Information has been obtained which suggests that frequency of occurrence of these agents in uninoculated cultures may be relatively high. Thus in 6 of 9 series of cultures prepared from kidneys of different monkeys one or more of 7 to 41 cultures in each series held 20 to 55 days showed characteristic degeneration (Table I); and the recovery of an agent from cultures of 3 different series was successful. One attempt to recover an agent from uninoculated cultures of a kidney series which did not show degeneration was unsuccessful after 3 blind passages in HeLa cells and subsequent passage in monkey kidney cultures. We have consistently observed in practically all cultures prepared from different monkey kidneys occasional small vacuoles apparent from the onset of cell outgrowth. In some cultures they persisted, but in others they apparently disappeared with continued incubation.

Infectivity titer in HeLa and monkey kidney cultures. Infectivity titrations were made with 2 of the 4 agents in HeLa cell and monkey kidney cultures. Ten-fold dilutions of fluids from infected cultures were prepared and 0.1 ml of each dilution inoculated into 4 cultures. The TC_{50} was estimated by the method of moving averages(11). Titration in HeLa cells of HeLa cell line of agent MK-D (previously passed serially 8 times in monkey kidney cultures) in passage 2,3,4 and 8, gave infectivity titers of $10^{-3.0}$, $10^{-2.8}$, and $10^{-3.5}$ respectively. Eleventh monkey kidney passage fluid of the same agent titered to $10^{-3.0}$ in monkey kidney cultures. Four additional titrations were made with the same and earlier passage fluids of the monkey kidney line in monkey kidney cultures. In 3 of these no estimation of infectivity titer was possible because of occurrence of characteristic degeneration in uninoculated controls. It might be noted that agents MK1 and MK4 were recovered in HeLa cell cultures from 2 different series of these control cultures. In the 4th bacterial contamination occurred. Two titrations were also carried out with MK3 agent in HeLa cell cultures. Third HeLa cell passage fluid gave a titer of $10^{-1.0}$ but after 5 additional passages, it increased to $10^{-2.8}$.

Effect of agent MK-D in other hosts. No obvious signs of disease were produced in 30 to 60 days with agent MK-D in its 8th to 12th monkey kidney tissue culture passage in 8 monkeys and 8 rabbits inoculated intracerebrally (0.6 to 0.8 ml) and intracutaneously (2 ml) or intraperitoneally (5 ml) and intramuscularly (2 ml). Intraperitoneal inoculation (1 ml) of one-day-old chicks and intracerebral inoculation (.03 ml) of young adult mice also failed to elicit obvious reactions. No lesions were formed on chorioallantoic membrane of 10- to 12-day-old chick embryos. However, in 2- to 3-day-old mice inoculated intracerebrally occasional deaths occurred with early and late passage fluids of the agent in monkey kidney culture. Blind passage experiments were then made to determine if more uniform results could be obtained. Following intracerebral inoculation of 7th passage fluid, 4 infant mice were killed after 7 days. Of the remaining 12 mice, 9 died within 3 weeks. Brains of 4 killed mice were passed as a 20% suspension (10% rabbit serum-saline) immediately after harvesting. With this second passage 5 mice were sacrificed at 7 days and of remaining 13, four were dead in 3 weeks. By the 4th rapid serial passage all of 17 infant mice inoculated were moribund or dead in 3 days. The brains of a number of these sick mice were passed with the same result. But with the next passage (6th) the incubation period increased to 10 days. Inoculations were repeated with 5th passage brain suspension and this time no infection occurred, and 3 blind passages with this suspension gave the same results. Fourth passage brain suspension was then tested again and it also failed to cause reactions. These findings have not been explained. However, in contrast to previous brain suspensions which were prepared and passed immediately after harvesting the brains, 5th passage brains had been stored for a month at –20°C prior to their passage. The second experiment with 4th passage brain suspension was made after 4 months storage in a CO_2 cabinet. It is possible that these relatively long storage periods may have been detrimental to subsequent activity of the agent in infant mice.

Other characteristics of agents. No bacterial growth occurred following inoculation of blood agar, veal infusion, and sodium thioglycollate broth with 2 of the 4 agents. No *Leptospira* were cultured after inoculation of agent MK-D in Fletcher's medium and baby chicks(12). Studies for pleuropneumonia organisms were kindly carried out by Dr. L. Dienes of the Massachusetts General Hospital, Boston, with negative results. *Agent MK-D* passed through a Selas (.03 porosity) but not through a Seitz (fine) filter. The Selas filtrate titered to 10^{-2} as compared to 10^{-3} of unfiltered control. Both monkey kidney and HeLa cell lines of agent MK-D were infectious for at least 4 months and agents MK1 and MK3 at least 2 months after storage in a CO_2 cabinet. After such storage and even for shorter periods, incubation periods were prolonged almost a week in some instances. Monkey kidney line of agent MK-D did not show as much variation in incubation period under these conditions as did its HeLa cell line or the HeLa cell lines of agents MK1, MK3 and MK4. In addition, the monkey kidney line of agent MK-D was stable, with no significant variation in incubation period, for at least 2 days at room temperature. However, after heating at 56°C for 30 minutes it failed to produce cytopathic effects in monkey kidney cultures for 21 days. The same results were obtained for agent MK4 in HeLa cell cultures.

Neutralization studies. Neutralization tests were made with agent MK-D and normal sera from rhesus monkeys, other animals and man. In addition, tests were performed with 2 lots of human gamma globulin and 1 lot of bovine and porcine gamma globulin and with sera prepared against a number of known viruses.¶ Tests were carried out by mixing an equal volume of undiluted fluid from infected HeLa cell cultures containing around 1000 to 3200 TC_{50} of agent with undiluted inactivated (56°C for 30 minutes) serum and 16% solutions of gamma globulins. In a few instances tests were made with monkey kidney line of same agent. Following incubation 1 hour at 37°C, 0.1 ml of each agent-serum mixture was inoculated into 3 or 4 HeLa cell or monkey kidney

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¶ Human gamma globulins were kindly supplied by Dr. F. F. Johnson, Cutter Laboratories and porcine and bovine gamma globulins by Dr. Lawrence L. Lachat, Armour and Co.; rabbit "anti B" serum by Dr. Albert B. Sabin, guinea pig anti-LCM serum by Dr. Joel Warren and mouse anti-yellow fever serum and monkey anti-Sinbis, West Nile and Japanese B serums, by Dr. Max Theiler.

TABLE II. Neutralization Results with Agent D Tested against Normal Sera and Various Immune Sera.

Sera	Results, No. positive/No. tested	Range of incubation period
A. Agent controls	9/9	6 to 9 days
B. Normal		
Monkey	16/29	pos 13.7 to ∞* neg 6 to 11.3
" pool	1/1	>14
Human	0/20	7 to 9
" pool	0/1	7
" γ globulin	0/2	6 to 7
Rabbit	0/9	7 to 10
Mouse pool	0/3	6.3 to 8
Horse "	0/2	7 to 9
Chicken "	0/1	8
Bovine γ globulin	0/1	6
Porcine γ globulin	0/1	8
C. Immune		
1. Non-monkey		
Sabin B, yellow fever, LCM, dengue (Hawaii and Saigon), Columbia SK, GDVII, FA, Influenza A and B	0/1 (each type)	6.5 to 9
2. Monkey		
Sinbis	0/2	7 to 9
West Nile	1/1†	14.3
Japanese B	1/1†	13.7

* No cytopathic effect after 21 days.

† Delayed cytopathic effect obtained with these 2 sera was due, in all probability to the normal component since normal sera from these monkeys caused the same delay in reaction.

cultures. The agent, mixed with an equal volume of fresh fluid, and held under same conditions served as control. Limited tests were also made with other agents and normal monkey sera. A relatively large number of normal monkey sera, including some collected 3 years ago, caused a significant delay in development of cytopathic effect of agent MK-D (Table II). However, the neutralization effect with different sera varied, ranging from a delay of 5 days in average incubation period to complete neutralization for at least 21 days. In contrast, none of the normal sera from other species gamma globulins, and immune serums caused any significant neutralization. Five normal monkey sera which neutralized agent MK-D and 5 which failed to do so were tested against agent MK3 with the same results. Normal monkey serum pool neutralized all 4 agents. These findings provide evidence that the agents were derived from renal tissue of monkeys. Evidence that other tissue culture constituents are not the source of the agent is as follows: One agent was recovered at time Hanks-Simms solution was used instead of bovine amniotic fluid and the reverse was true for other 3 agents. Beef embryo extract has been routinely employed for cultivation of HeLa cells for over a year without resulting in progressive characteristic degeneration which occurred following inoculation of such cultures with the agents. Horse serum has been heated at 56°C for 30 minutes prior to its use as a medium constituent, and, as previously noted, 2 of the 4 agents so treated failed to produce cytopathic effects after 21 days. Finally 3 blind passages in HeLa cells and subsequent passage in monkey kidney cultures of fluids from one monkey kidney series failed to reveal an agent. But agents were recovered from cultures of 2 other monkey kidney series set up shortly prior to and after this series with the same lots of medium constituents.

Failure of MK-D agent to interfere with poliomyelitis viruses in monkey kidney cultures. In view of the importance of monkey kidney cultures in the study of poliomyelitis (13), an experiment was carried out to determine if agent MK-D would interfere with infectivity of poliomyelitis viruses in tissue culture. A number of 10-day-old monkey kidney cultures were inoculated with freshly harvested MK-D agent in its 14th monkey kidney passage. After 5 days definite foci of syncytial masses and vacuolation were present. Four such cultures were then inoculated with 3.2 TC_{50} and 4 additional cultures with 10 TC_{50} of type 2 virus, 4 each with 20 and 60 TC_{50} of type 1, and 4 each with 20 and 60 TC_{50} of type 3 poliomyelitis viruses. Rounding degeneration, characteristic of poliomyelitis viruses, was evident with no delay even in areas in close proximity to syncytial and vacuolation reactions. Moreover, the amounts of each type of poliomyelitis virus employed did not appear to interfere with further development of reaction by agent MK-D. In a number of cultures the reaction had spread further with appearance of rounding degeneration.

Further incubation resulted in complete rounding degeneration and disappearance of cells with only an occasional vacuolated area visible.

Summary. During attempts to adapt dengue virus to rhesus monkey kidney cultures, an unidentified agent which causes formation of syncytial masses and vacuolation in such cultures was encountered. Subsequently, 3 additional agents with similar cytopathogenic effects were passed and maintained in HeLa cell cultures from uninoculated monkey kidney cultures. Renal tissue and not the medium constituents is the source of the agent. Bacteriological studies with one agent were negative. The same agent passed through a Selas filter. Accordingly it is considered to be virus-like in nature. Similar experiments were not done with 3 other agents but because of certain common characteristics are believed to be of the same nature.

The authors are grateful to Mr. Joseph Joyner and Mr. Oliver Rampersaad for their technical assistance in certain phases of this study.

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In vivo Method for Studying Intestinal Motility in the Rabbit.* (21479)

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(Introduced by R. W. Wilkins.)

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The purpose of this report is to describe a method devised to record the effects of ionizing radiation on the intestinal motility of the rabbit. We believe that this method offers the following advantages: (1) anesthesia is avoided, (2) comparable, repeated studies are possible in the same animal, (3) the gut is in the usual position and its continuity is maintained, and (4) intra-enteric pressure is measured directly and can be differentiated from intra-peritoneal pressure.

Operative Procedure. Male white rabbits (2-3 kg) were used. The animals were anesthetized with pentothal sodium (30 mg/kg), administered intraperitoneally. A 12 cm midline ventral skin incision was made. Three polyethylene tubes (int. diam. .78 mm, ext. diam. 1.22 mm) were passed subcutaneously with a Kelly clamp from the abdominal incision to the back of the neck. An incision was made in the skin of the neck and the tips of the tubes exteriorized. Each tip was secured by forcing one arm of a U-shaped pin into the tube; the other arm was attached to a plastic sheet which was sutured to the skin. The pins were easily detached when the tubes were used for recording. The abdominal skin incision was then extended into the peritoneal cavity. A stab wound was made in the lesser curvature of the stomach, approximately 1 cm from the

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* This study was performed under contract with the U. S. Atomic Energy Commission.

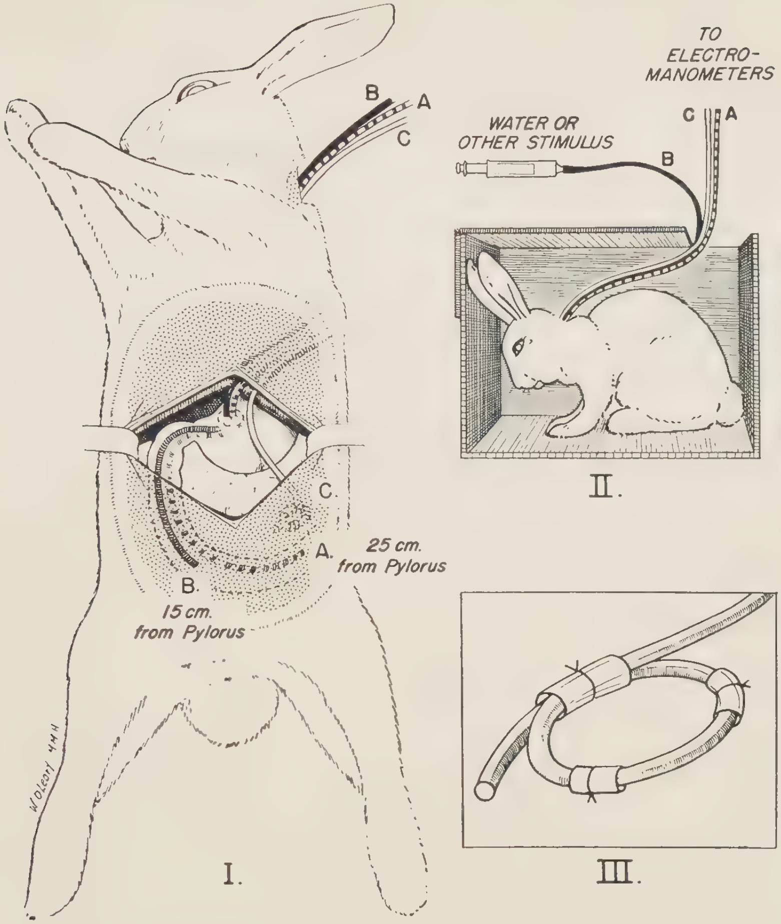

FIG. 1. Diagrams showing operative and recording procedures. I. Demonstrates position of tubes A (for intraenteric recording), B (for delivering the intraluminal stimuli) and C (for intraperitoneal recording). II. Shows position of rabbit during recording of intestinal motility. III. Shows details of terminal loop of tube C.

pylorus. Two tubes (A and B, Fig. 1, I) were joined together side by side by encircling them at several points with short segments of large-bore polyethylene tubing. They were then passed into the intestine via the gastrostomy so that the tip of one tube was 15 cm distal and the tip of the other 25 cm distal to the pylorus. At a point 3 cm distal to the pylorus a retaining ligature was passed through the intestinal wall securing the 2 tubes to the duodenum. The gastrostomy opening was closed tightly around the tubes with a purse-string suture. The third tube (C) was affixed to the posterior peritoneum in the approximate region of the distal intestinal tip (Fig. 1, III). The loop shown in the diagram was necessary to prevent the tube from pulling out. The abdominal muscle and fascia were closed about the 3 tubes, and the skin closed over them. The animals received prophylactic penicillin and were returned to their cages. After recovery from the operation, the animals appeared to tolerate the inlying tubes without difficulty, as evidenced by normal activity, adequate food intake and continuous weight gain over a period of several months.

Recording Procedure. Motility studies were carried out 24 hours or more after operation. After operation and between studies, food was given *ad libitum*. During recording,

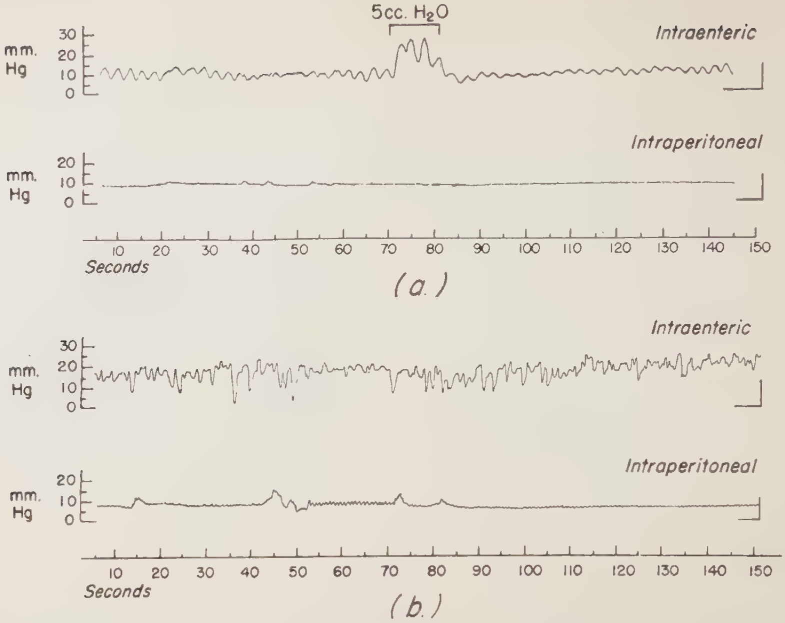

FIG. 2. Intraenteric pressure recordings in normal rabbits. (a) Effects of introducing 5 cc of water through proximal intestinal tube. (b) Spontaneous fluctuations in intraenteric pressures.

the animal sat in a non-restraining box which shielded the operator from the animal's vision (Fig. 1, II). In several experiments, a standard rabbit box was used instead. The proximal intestinal tube (B) was used to deliver mechanical or pharmacological stimuli to the intestine. The distal intestinal tube recorded intra-luminal pressures, and the peritoneal tube recorded respirations, struggling, and other changes in intraperitoneal pressure. By this means effects of these extra-intestinal pressures on the intra-luminal pressure record could be recognized. When the effects of parenteral drugs on intestinal motility were studied, 21-gauge needles were inserted subcutaneously or intravenously (ear vein) prior to recording, in order to prevent the concomitant struggling from appearing on the intestinal record. No effort was made to calibrate the recordings in terms of absolute pressures. Plugging of either the intraluminal or intraperitoneal tube was infrequent. When this

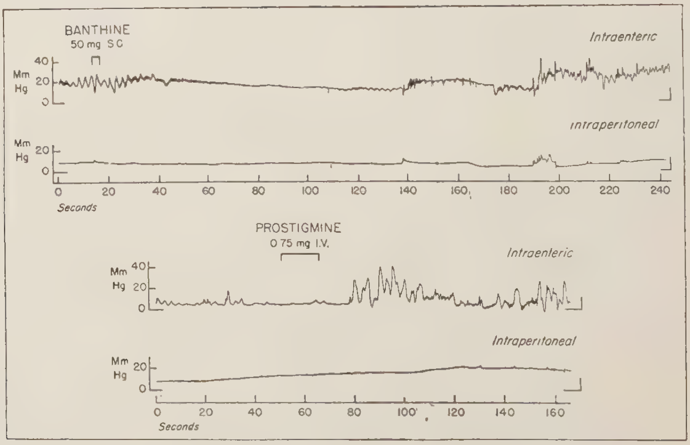

FIG. 3. Effect of drugs on intraenteric pressures in the rabbit.

occurred, its effect on the record was readily recognized and was corrected by irrigating the catheter with saline.

The method has been used successfully in the study of the intestinal motor activity of 22 rabbits. Two examples of the pressure patterns recorded are shown in Fig. 2. Fig. 3 shows that the pressure record responds in a clear-cut manner to the administration of drugs. These responses to pharmacological agents of known action indicate that the records obtained satisfactorily reflect intestinal motility.

Summary. A method is described for recording the intestinal motility of an unanesthetized rabbit. Frequent comparable studies can be obtained in the same animal. Simultaneous recording of intraperitoneal pressures permits separation of intrinsic intestinal pressures from extra-intestinal effects. The intestinal motor activity, as recorded by this method, responds to intra-luminal mechanical stimulation and to pharmacological agents.

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Correlation of Emetic and Hypotensive Doses of Veratrum in Pigeon, Dog and Human.* (21480)

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The emetic properties of veratrum derivatives have been recognized since the time of Hippocrates. Christensen and McLean(2) and Jenkens and Christensen(4) suggested that the emetic action in the pigeon be used as a basis of a method of assay of veratrum. Their work, however, was limited to tinctures of veratrum and did not include any of the purified extracts or alkaloids of veratrum. Swiss(10) suggested the possibility of a correlation between the emetic and hypotensive actions of 21 alkaloids and alkaloidal mixtures in the dog. Abreu *et al.*(1) observed a relationship between hypotensive and emetic activity for protoveratrine A, Veralba and Unitensen.

The present investigation of purified veratrum derivatives was undertaken to determine the correlation of (a) emetic activity and hypotensive activity in the dog (b) emetic activity in the pigeon with the hypotensive activity in the dog (c) the emetic activity in the dog with the emetic activity in the pigeon and (d) the results of bioassay in the dog and human response.

Method. Alkavervir (Veriloid)† was dissolved in 2 ml of a mixture of equal parts of propylene glycol and ethyl alcohol and then diluted to the final volume with 0.9% sodium chloride solution containing 0.1% acetic acid. The purified alkaloids‡ were dissolved in several ml of 5% acetic acid and then diluted to the final volume with 0.9% sodium chloride solution and acetic acid so that the final solution contained 0.1% acetic acid. Fifteen to 45 dogs were utilized in the determination of the 50% emetic dose (ED_{50}) for each veratrum derivative. Each was administered into the foreleg vein during a period of 30 seconds.

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* Grateful acknowledgement is made to Riker Laboratories, Los Angeles, Calif. for financial support.

† Veriloid (trademark of Riker Laboratories) is a biologically standardized alkaloidal extract of veratrum viride of uniform hypotensive potency.

‡ Substances utilized were kindly provided by following sources: (a) Eli Lilly and Co., Indianapolis, Ind., protoveratrine P49 366. (b) S. B. Penick and Co., Detroit, Mich., veratrine. (c) Dr. S. J. Ringel, Bureau of Entomology and Plant Quarantine, U. S. Dept. of Agric., Beltsville, Md., cevadine (d) Riker Laboratories, Los Angeles, Calif., protoveratrine from Veratrum viride, neogermitrine, neoprotoveratrine, escholerine, Veriloid, and veratridine. (e) Squibb Institute for Medical Research, New Brunswick, N. J., germitrine, germidine, germerine and neogermitrine.

TABLE I. Comparison of Hypotensive Dose* and 50% Emetic Dose (ED_{50}) in Dogs.

Drug, lot No. (MP°C)†	ED_{50} (μg/kg) 19/20 C.L.‡	Hypotensive dose (μg/kg/min.) 19/20 C.L.
Neogermitrine JF115C5 (231-232°C)	1.9 (1.3- 2.8)	.115 (.099- .136)
Germitrine JF97617	2.0 (1.6- 2.6)	.079 (.069- .92)
PNP 111	2.5 (1.9- 3.2)	.094 (.086- .104)
Protoveratrine-veratrum viride 3031 (262°C)	3.4 (2.4- 4.8)	.185 (.148- .246)
Neogermitrine 3062 (234°C)	3.5 (2.4- 5.1)	.134 (.119- .153)
Germerine JF97ABC3 (199-201°C)	4.2 (2.5- 6.0)	.189 (.168- .217)
Neoprotoveratrine 3085 (246°C)	4.4 (3.3- 5.8)	.256 (.211- .324)
Protoveratrine Lilly P49 366 (246°C)	5.0 (3.2- 7.7)	.211 (.195- .230)
Germidine JF6868 (230-232°C)	8.1 (7.1- 9.2)	.415 (.364- .483)
Escholerine	10.8 (8.7- 17.3)	.42 (.364- .51)
Veriloid	18.8 (16.1- 21.9)	1.0 (.92 -1.1)
Veratridine (171°C)	46.0 (31.9- 66.2)	2.0 (1.72 -2.38)
Veratrine 1801LGB	77.0 (65 - 92)	3.39 (2.92 -4.05)
Cevadine (209°C)	110 (81 -149)	5.52 (4.34 -7.69)

* These data are from Maison, Gotz and Stutzman(7). Hypotensive dose is estimate of μg/kg/min. over a 10 min. period required to produce an average fall of mean arterial pressure of 30% in a large number of anesthetized dogs.

† MP°C = Melting point in degrees C.

‡ 19/20 C.L. = 19/20 confidence limits.

Each animal was observed for one hour although all the emetic responses occurred within the first 20 minutes. Solvent controls were performed. Adult pigeons (450-600 g) were weighed to the nearest gram. Intravenous injections were made into a wing vein during a 30 second period. The animals were observed for 2 hours. No animal received more than one injection per week. Each derivative was tested on a series of 10 pigeons. Solvent controls were performed for each derivative tested. The ED_{50} values were calculated by the method of Litchfield and Wilcoxon(6). The hypotensive doses for the dog were determined by the method of Maison, Gotz and Stutzman(7). The hypotensive responses of the human were obtained from the clinical studies of protoveratrine by Meilman and Krayer(8), Veriloid by Stearns and Ellis(9), germitrine, germidine, and germerine by Freis, Stanton and Moister(3).

Results. Table I contains the ED_{50} values and the hypotensive dose§ in dogs(7) for each veratrum derivative. The relationship between the emetic dose in dogs and the hypotensive dose in dogs is depicted in Fig. 1 in which the regression equation and the coefficient of correlation ($r_{yx} = 0.926$) indicate the high degree of correlation of these 2 variables(5).

The data on the emetic activity in the pigeon of 6 different veratrum derivatives are contained in Table II. There is a close correlation between the emetic dose in pigeons and the emetic dose in dogs as well as between the emetic dose in pigeons and the hypotensive dose in dogs as shown by regression lines (Fig. 2). Control injections of the solvent in no case produced emesis.

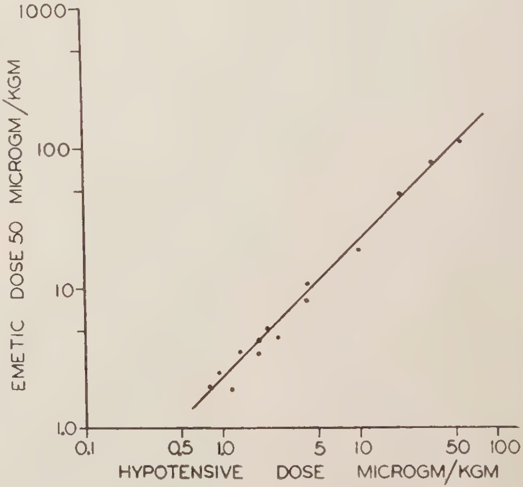

FIG. 1. Correlation of emetic dose 50 and hypotensive dose in dogs. Hypotensive dose is estimate of μg/kg over a 10 min. period required to produce average fall in mean arterial pressure of 30% in anesthetized dogs. Regression equation was calculated to be H.D. = 0.48 (ED_{50}) − 0.20.

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§ Hypotensive dose in dogs is estimate of μg/kg/min. over a 10 min. period required to produced average fall of mean arterial pressure of 30% in anesthetized dogs.

TABLE II. Correlation of Pigeon Emetic Dose 50 and Dog Hypotensive Dose.*

Veratrum derivative (MP°C)†	Pigeon emetic dose 50, μg/kg (19/20 C.L.)‡	Dog emetic dose 50, μg/kg (19/20 C.L.)	Dog hypotensive dose, μg/kg/min. (19/20 C.L.)
Protoveratrine 3031 (262°C)	6.0 (4.7- 7.6)	3.4 (2.4- 4.8)	.19 (.15- .25)
Neogermitrine 3062 (234°C)	7.0 (4.4- 9.8)	3.5 (3.3- 5.8)	.134(.12- .15)
Escholerine	18.0 (10 - 32)	10.8 (8.7-13.4)	.42 (.36- .51)
Veriloid subsidiary standard	31. (25 - 39)	15.2 (12.2-18.4)	.66 (.54- .82)
Veratridine (171°C)	73. (55 - 97)	46. (32 -66)	2.0 (1.7 -2.4)
Veratrine 1801 LGB	182. (135 -240)	77. (65 -92)	3.4 (2.9 -4.1)

* Dog hypotensive dose is estimate of μg/kg/min. over a 10 min. period required to produce average fall of mean arterial pressure of 30% in anesthetized dogs.

† MP°C = Melting point in degrees C.

‡ 19/20 C.L. = 19/20 confidence limits.

Discussion. The high degree of correlation between emetic dose and hypotensive dose (Fig. 1) in the dog indicates the practicability of an assay method for veratrum based on the emetic response of the dog as previously suggested by Swiss(10). From the clinical reports of Stearns and Ellis(9) and Meilman and Krayer(8), it appears that clinically effective doses of Veriloid or protoveratrine in man are approximately equivalent to the dog hypotensive doses found by Maison, Gotz and Stutzman(7). This correlation offers further support for the dog assay method. Similar conclusions may be drawn from the results of the clinical study of germitrine, germidine and germerine by Freis, Stanton and Moister(3), although the data are less convincing because of the limited number of experiments performed. It thus appears reasonable that the emetic dose in dogs might be useful in predicting the dosage range for human administration as well as for predicting the relative potencies of veratrum derivatives. One of the advantages of the emetic assay method lies in the fact that the animal may be used many times at intervals of several days. Swiss(10) has shown that the threshhold emetic dose for an individual dog does not change after repeated injections of a veratrum derivative; therefore in a given series of dogs the response to an un-

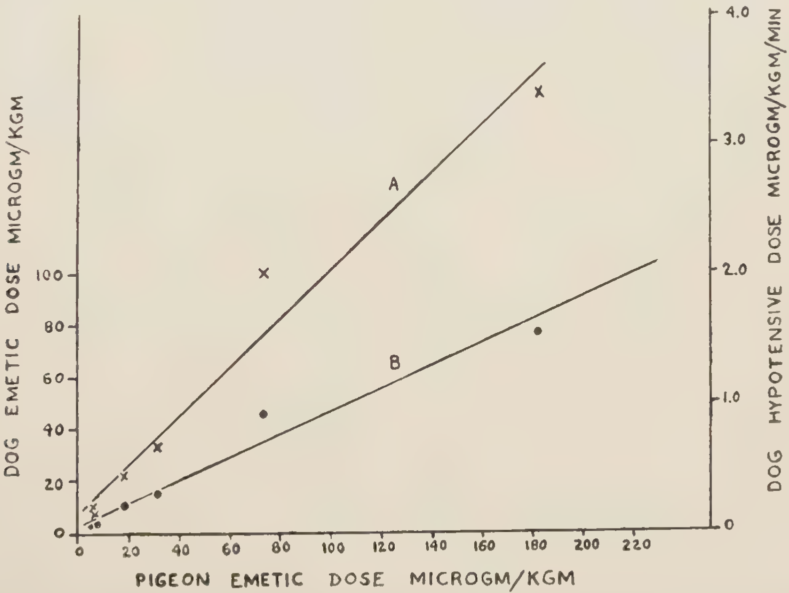

FIG. 2. Regression lines: (A) pigeon 50% emetic dose versus dog hypotensive dose. (B) pigeon 50% emetic dose versus dog 50% emetic dose. Regression equation for line A was calculated to be $y = 0.019x + 0.14$. Regression equation for line B calculated to be $y = 0.44x + 2.56$.

known veratrum derivative may be compared to the response of a standard preparation in the same animal. The avoidance of anesthesia, the occurrence of the emetic response within 20 minutes after intravenous injection and the fact that within wide limits the response is not influenced by the rate of administration may be considered advantageous. Once the ED_{50} value has been determined, the hypotensive potency for the derivative can easily be estimated by reading from the line presented in Fig. 1, the hypotensive dose corresponding to the ED_{50} value.

The results of these experiments further substantiate the work of Christensen and McLean(2) and Jenkins and Christensen(4) on tincture of veratrum in that the correlation between the emetic activity in the pigeon and the emetic as well as the hypotensive activity in the dog has been demonstrated for 6 different purified derivatives of veratrum.

The emetic activity of veratrum, although different from the activity which finds clinical application may be useful in the laboratory as the basis for a preliminary rapid economical method of assay.

Summary. A close correlation between the emetic activity and hypotensive activity in the dog has been demonstrated for 14 purified veratrum derivatives. A correlation has also been shown to exist between the emetic activity in the pigeon and the emetic and hypotensive activity in the dog. The emetic activity of veratrum in dogs is suggested as a method of assay.

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Isolation and Type Determination of Coxsackie Virus, Group B, in Tissue Culture. (21481)

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Strains of Coxsackie virus Group B have been, in general, more difficult to isolate and identify than those of Group A. The very narrow age range, together with the less apparent signs of disease and the frequently longer incubation period, are disadvantages connected with use of suckling mice. Many more hindrances have also presented themselves in Group B complement-fixation tests than with Group A. An *in vitro* method for the isolation and type determination of Group-B strains is now suggested by their cytopathogenicity for trypsinized monkey kidney cells. The earliest isolations of cytopathogenic Coxsackie virus were Group-A strains, Type 9 (1,2). Several investigators have, however, reported growth or cytopathogenicity of Group-B strains on a variety of tissues, including mouse interscapular fat pad tissue(3), mouse skeletal muscle(4), human embryonic brain and intestine and mature kidney(5), HeLa cells(6,7), and monkey kidney cells (8,9).

Materials and methods. Human uterine tissue. Plasma clot cultures of human uterine tissue were prepared, in general, according to

TABLE I. Neutralization Tests in Tissue Culture. Coxsackie virus Group B, Types 1 and 4.

Test dose			B-1 R48-1039				B-4 R51-336			
	Serum									
Virus	Kind	Dilution	3 days		6 days		3 days		6 days	
.5 × 10^{-2}	Medium 199H		±	+	+	2+	±	+	2+	2+
× 10^{-3}			—	—	+	2+	±	?	+	+
× 10^{-4}			—	—	+	+	—	—	±	+
× 10^{-5}			—	—	—	—	—	—	—	—
.5 × 10^{-1}	Normal hamster	5	+	+	2+	2+	+	+	2+	2+
	B-1	5	—	—	—	—	+	+	2+	2+
		40	—	—	—	—	0		0	
		80	—	—	?	?	0		0	
	B-2	5	±	+	+	+	±	+	+	2+
	B-3	5	+	+	2+	+	+	+	+	2+
	B-4	5	+	+	2+	2+	—	—	—	—
		10	0		0		—	—	—	—
		20	0		0		—	—	—	—
		40	0		0		—	—	—	?
		80	0		0		—	—	±	±
		160	0		0		—	—	+	±
	B-5	5	+	+	2+	2+	±	?	2+	+
Uninoculated controls			—		—		—		—	

0 = not done; — = same as control uninoculated tissue; ? = slight damage, specificity doubtful; ± = small but definite area of damage; + = large areas of definite damage but with large remaining areas of normal cells; 2+ = extensive destruction.

the method of Robbins, Weller, and Enders (10). The nutrient fluid consisted of 5% chick embryo extract, 10% heated horse serum, and 85% ox serum ultrafiltrate-Hank's solution. For the test maintenance fluid the horse serum content was reduced to 5%. To both were added 50 units each of penicillin and streptomycin. Mixture 199(11) was also used as a maintenance fluid. Cultures were placed horizontally in cake pans with the tube neck slightly raised, and incubated, stationary, at 35°-36°C. *Trypsinized monkey kidney cells.* The method followed in general those of Dulbecco(12) and Youngner(13). Mixture 199 (Hank's) plus 2% heated horse serum was used as the nutrient fluid; mixture 199 (Hank's or Earle's) alone as test maintenance fluid. Incubation was stationary at 35°-36°C. Monkey kidney cell cultures may also be purchased from Microbiological Associates, Inc. *Isolation.* Fecal suspensions (20%) were prepared in physiologic salt solution to which 10% infusion broth had been added. Instead of ether an antibiotic mixture was used, 500 units of penicillin and 2.5 mg streptomycin per ml. After replacing the nutrient fluid on the cultures with mixture 199, each of three tubes was inoculated with 0.1 ml of the fecal suspension previously diluted one-half with the same medium. The tubes were incubated as above. Uninoculated tissue controls were included in the test. *Neutralization.* To determine the cytopathogenicity of the test virus, tubes were inoculated with 3 or more 10-fold dilutions combined with an equal amount of Hank's solution or mixture 199. Controls consisted of pooled normal sera from the same animal species as the immune sera and also uninoculated tissue culture tubes. For screening tests, normal and Group-B sera were used diluted 1:5 with Hank's solution against approximately 100-1000 infectious doses of virus; for confirmatory test, 2-fold dilutions of specific serum through 1:80 or 1:160. Incubation was the same as with uterine tissue. In preparing the test, the fluid on the monkey kidney cell cultures was replaced with mixture 199. Equal parts of virus dilution and serum dilution were mixed and held at room temperature for one hour. Three monkey kidney cell tubes were inoculated with each mixture (0.1 ml each) and test and uninoculated controls examined daily for cell destruction. The tests could usually be terminated within a week.

Results. Human uterine tissue. Twenty-four strains of Coxsackie virus Group B in suckling mouse brain or leg suspension were tested on human uterine tissue. They were distributed as follows: Six strains of Type 1, 5 of Type 2, 10 of Type 3, 2 of Type 4, and 1 of Type 5. Of these, cell destruction was noted with only 4, 3 strains of B-3 and the B-5 strain. The B-3 strains included the type representative Nancy, a strain from an outbreak of pleurodynia in the State of Washington(14), and a B-3 strain received as a mixture with A-14 and separated by serologic means. Cell damage by the B-3 strains was slower and less complete on the uterine tissue than on monkey kidney tissue and the infectivity titer lower. Beginning activity was noted usually on the 5th to 7th day, occasionally earlier, but as late as the 9th day. The B-5 virus was isolated by Dr. Alex J. Steigman(8) who found it cytopathogenic in renal epithelium cultures. With this strain cell destruction of human uterine tissue was earlier and more marked than by the B-3 strains but less than on monkey kidney cells. All were neutralized by their specific sera.

Monkey kidney cells. A similar number and type distribution of strains in infected infant mouse tissue were tested on monkey kidney cell cultures. All were cytopathogenic. One was unusually slow. Kidney cell cultures of pooled mouse brain suspension of the 35th and 36th mouse passage of the B-1 representative strain did not show cell damage until the 8th day in the second generation. The suspensions were highly virulent for suckling mice. This same line was described by Dalldorf and Gifford as failing to induce pancreatic necrosis in adult mice(15). However, in the original fecal specimen the strain was typically cytopathogenic and could be readily reisolated on monkey kidney cells.

Isolation of cytopathogenic Group-B virus from fecal suspensions was made from 21 additional specimens including 4 Type 1, 11 Type 2, 2 Type 3, and 4 Type 4.

The preliminary incubation period was in general longer than is usual for poliomyelitis virus, the endpoint titers lower, and the degree of cell damage less complete. Cell damage may be noted in from 3 to 7 days with maximum effect at from 4 to 9 days. Table I records neutralization tests of strains isolated from feces on monkey kidney cells. The tests are the third generation. Screening at the time of isolation may also be done by incubation of portions of the specimen with the various immune sera.

Summary. Suckling mouse tissue suspensions of strains of Coxsackie virus Group B, Types 1-5, have been tested on trypsinized monkey kidney cells. All types were cytopathogenic. Strains of Group B, Types 1-4, have been isolated from fecal specimens on monkey kidney cells and carried through type determination by the neutralization method in monkey kidney cell cultures.

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Synthesis of Hexosamine in the Rat. (21482)

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The synthesis of hexosamine* by bacteria (1,2), laying hens(3), and rats(4) has been demonstrated by tracer technics utilizing C^{14}-labeled glucose. Cultures of rabbit connective tissue have also been shown to synthesize hexosamine(5). In the experiments reported here, balance studies were carried out on rats to determine the net amount of hexosamine synthesis by the intact animal during a period of active growth. To investigate the role of dietary hexosamine in these studies various dietary levels of hexosamine were employed. The effect of fasting on the hexosamine content of the rat was also studied. As a preliminary step the quantitative changes in total carcass hexosamine content during the growth period were determined to enable the selection of a period of maximal hexosamine accumulation.

Methods. Male Sprague-Dawley rats of closely similar body weight were used in each experiment and were permitted to drink water and eat *ad libitum.* The rats were kept individually in cages with ½″ wire mesh flooring. In the hexosamine balance studies, the excreta were collected on paper sheets beneath each cage and screened later to separate the feces from the spilled food. The rats were killed by etherization. The contents of the urinary bladder, stomach and intestines were removed and added to any urine or feces previously eliminated by the same animal during the experimental period. In the growth study all of the urine, gut contents and feces were discarded. The term "carcass" as used throughout this paper refers to the entire body devoid of the contents of the urinary bladder, stomach and intestines. The samples for hexosamine analysis were hydrolyzed in stoppered Erlenmeyer flasks for 5 hours in a boiling water bath. The carcasses were hydrolyzed in volumes of 6*N* HCl equal numerically in ml to the carcass weights in grams. The feces were hydrolyzed with 4*N* HCl (volume varying with the amount of feces). The urines, absorbed on double sheets of Whatman No. 3 filter paper together with all the spilled diet, were hydrolyzed with 150 ml of 2*N* HCl. Samples of diets and their constituents were hydrolyzed with 1 ml of 4*N* HCl in stoppered 10 ml volumetric flasks at 100°C for 15 hours. The hydrolyzates were diluted with distilled water and filtered. Hexosamines were isolated from the hydrolyzates on Dowex-50 and determined by a modification(6) of the method of Elson and Morgan. Absorption spectra of the color produced by representative samples in the hexosamine method were determined with a Beckman Spectrophotometer (Model DU). All samples were found to be free of interfering chromogens.† Although muscle hydrolyzates contain interfering chromogens(6) that cannot be completely eliminated by Dowex-50, no interference was detected in the resin-treated carcass hydrolyzates because of the large dilution involved. Filter paper contained no hexosamine or interfering chromogen.

Results. Carcass hexosamine and growth. The total carcass hexosamine content was determined in seven groups of rats at 0, 4, 10, 20, 40, 80, and 160 days of age. Each group consisted of 5 rats allocated so that there were not more than 2 litter-mates per group. The rats 20 days of age and younger were sacrificed on the day of separation from their mothers. The rats in the older groups were fed a stock diet, No. 4164(7), from the day of weaning until

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* The only naturally occurring hexosamines are glucosamine and galactosamine. The method used in this paper does not distinguish between these 2 amino sugars.

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† The hexosamine content of one of the diets used, No. 10,040, was calculated from the concentrations in each of its constituents. Determination on the diet itself was not feasible due to an interfering chromogen possibly formed by interaction of the constituents, since it was not detected in individual components.

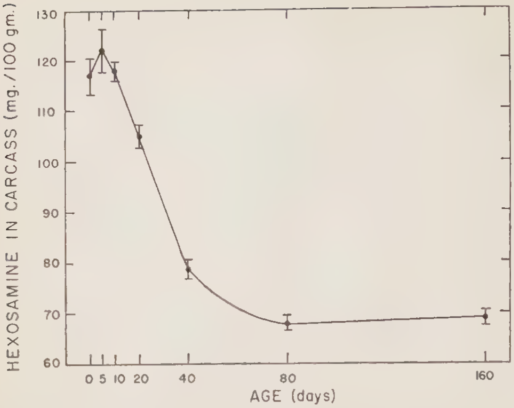

FIG. 1. Effect of age on hexosamine content of carcass. Vertical lines represent S.E. of the means.

they were sacrificed. Body weights averaged 6.1 ± .2 g on the day of birth and 390 ± 13 g at 160 days of age, at which time the animals were still growing slowly. The total amount of carcass hexosamine increased in each succeeding age group from 6.9 ± .3 mg at birth to 260 ± 3.2 mg at 160 days. However, the concentration of hexosamine (mg/100 g carcass) decreased markedly from the 10th to the 80th day of age, following which there was no further change despite the continued increase in body weight (Fig. 1).

Fasting. The effect of fasting on carcass hexosamine content was studied in Exp. I. Ten rats, previously maintained on a supplemented Purina Laboratory Chow ration, were used. A control group (5 rats) was sacrificed at the start of the experiment and a starved group (5 rats) was killed after 3 days of total fasting with ample water available (Table I). The total carcass hexosamine content of the starved rats was 27% lower than that of the controls. Due to an even greater difference in the body weights of the 2 groups, the hexosamine concentration was higher in the starved rats, 97 mg/100 g carcass, as compared with 84 in the controls. The amount of hexosamine in the excreta and the contents of the gut and urinary bladder of the starved animals was 3 times higher than the amount present in the gut contents of the initial group. There was no significant net loss of hexosamine in the starved group relative to the controls when the gut contents and excreta were included, indicating that either no significant glucosamine destruction occurred or, if it did occur, such destruction was masked by synthesis.

Diets with varying hexosamine contents. In an attempt to formulate an adequate diet containing minimal quantities of hexosamine, the hexosamine contents (%) of various protein preparations were determined. They were as follows: zein (Argo, Lot No. 3307, Corn Prod. Refining Co.) .005; ground whole wheat, .03; crude casein (Borden Co.) .14; casein (General Biochemicals Inc.) .14; Labco casein (Borden Co.) .12; gelatin (Wilson Co.) .24; Soy, sodium proteinate (Archer-Daniels-Midland Co.) .29; Soy, alpha-proteinate (Glidden Co.) .33; and *Torula* (yeast) (Bradley and Baker) .99. On the basis of these analyses a zein diet No. 10,040‡ containing .0025% hexosamine was prepared.

Three groups of weanling rats containing 5 rats each were used in Exp. II (Table I): a control group sacrificed at the start of the experiment to determine the initial carcass hexosamine (Column 1); Group 1 fed diet 10,040; and Group 2 fed diet 10,040 to which had been added 70 mg of glucosamine-HCl/100 g of diet. Food consumption was determined by subtracting the amount of food spilled from the amount removed from the cup by the rat. All the feces, but not the urine, were collected. No change in body weight occurred during the 15-day experimental period. In Groups 1 and 2 the final carcass hexosamine contents were slightly less than the content of the control group (Column 1). Despite the fact that Group 2 rats consumed 38 mg more hexosamine than did Group 1 rats, the hexosamine contents in both groups were the same for the final carcasses and for the feces. The net hexosamine difference [(final carcass content + fecal output) — (initial carcass content + intake)] was + 38.0 mg in Group 1 and — .3 mg in group 2. The discrepancy between the net balances for the 2 groups was due to the added dietary glucosamine in Group 2. Thus the supplemented glucosamine could not be accounted for by the carcass and fecal analyses. The two possible explanations for its disappearance were urinary elimination or destruction within the rat.

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‡ Diet 10,040, a modification of diet B of Kligler and Krehl(8): 58.7% dextrin, white (Mallinckrodt); 25% Zein (Argo, Lot 3307, Corn Products Refining Co.); 8% cottonseed (Wesson) oil; 4% salt mixture H.M.W. (Nutritional Biochemicals Corp.); .3% l-arginine-HCl; .2% l-cystine; .2% l-histidine-HCl; 1% l-lysine-HCl; 1.2% dl-threonine; .3% dl-tryptophane; 1.6% dl-valine. Fat soluble vitamins (per kg diet): Natola (Parke, Davis & Co.), 350 mg/kg diet (supplying 19,250 USPU vit. A and 3,850 USPU vitamin D); 50 mg vitamin E and 1 mg vit. K (menadione). Water-soluble vitamins (mg/kg diet): thiamine-HCl, 2; riboflavin, 3; pyridoxine-HCl, 2.5; Ca-pantothenate, 20; choline chloride, 1000; inositol, 100; niacin, 20; biotin, .1; folic acid, 1.

Diet No. 4164, containing ground whole wheat, casein and whole milk powder, was employed in Exp. III (Table I). It contained .071% hexosamine. Three groups of weanling rats with 6 rats in each group were used: a control group sacrificed at the start of the experiment to determine the initial carcass hexosamine (Column 1); Group 3 receiving Diet No. 4164; and Group 4 receiving Diet No. 4164 to which had been added 84.2 mg of glucosamine-HCl per 100 g of diet. Body weight and total carcass hexosamine increased over 100%, and to the same extent for each factor, in Groups 3 and 4 during the experimental period. The total amounts of hexosamine in the intestinal contents, feces, and urine produced during the experiment were also the same in both groups, despite the doubled hexosamine intake for Group 4. This greater intake, therefore, was the reason for the smaller apparent synthesis or net hexosamine balance in Group 4. Thus, as in Exp. II, the glucosamine-HCl supplement in the diet could not be accounted for in the final carcasses or excreta. The possibility of any appreciable urinary elimination was discounted, since the hexosamine output in the urine and small amounts of waste food (3-5 g) was the same in Groups 3 and 4. Therefore, it seemed likely that the disappearance of this glucosamine was due largely to its destruction within the rat.

Discussion. The decrease in hexosamine concentration in the rat carcass observed during the rapid growth period was similar to that reported in rat skin(9) and connective tissue (10).

The balance studies demonstrated that

TABLE I. Hexosamine Balance Studies on Fasted Rats and on Rats Fed Diets with Various Hexosamine Contents.*

				Body wt (g)		Hexosamine (mg)					
Exp.	Group	Diet	Time (days)	Initial	Final	1 Initial carcass	2 Intake	3 Final carcass	4 Gut contents + feces	5 Urine + waste food	Net balance§
I	Control	—	0	98 ± 1.6	—	79 ± 2.5	—	—	6.8†	—	− 5.5
	Starved	—	3	97 ± 1.6	61 ± 1.6	—	0	58 ± 1.5	22 ± 2.4	—‡	
II	Control	—	0	41 ± .2	—	38 ± 1.0	—	—	—	—	—
	1	10,040	15	38 ± 1.1	36 ± .8	—	1.3 ± .1	33 ± 1.0	44 ± 1.1	—	+ 38 ± 1.4
	2	10,040 + glucosamine	15	39 ± .6	38 ± .9	—	39 ± 1.2	33 ± 1.1	44 ± 1.0	—	− .3 ± 1.1
III	Control	—	0	40 ± .3	—	34 ± .6	—	—	—	—	—
	3	4164	15	39 ± 1.2	105 ± 5.4	—	99 ± 5.3‖	78 ± 4.2	149 ± 6.0	33 ± 3.7	+127 ± 9.3
	4	4164 + glucosamine	15	39 ± 1.6	101 ± 5.6	—	194 ± 9.1‖	76 ± 3.6	153 ± 12.5	37 ± 2.7	+ 39 ± 10.4

* Data presented as means ± stand. errors. † Avg amount of hexosamine in gut contents. ‡ Urine included with feces. § Sum of columns 3, 4 and 5 minus sum of columns 1 (control group) and 2. ‖ Not corrected for spillage because waste food included with urine (column 5).

hexosamine is synthesized within the rat in amounts considerably in excess of its dietary intake. During a 2-week period shortly after weaning, twice as much hexosamine appeared in the carcass and excreta combined as could be accounted for by the original carcass and the food intake. This was true in the presence or absence of an increase in body weight on the diets not supplemented with glucosamine. The ability of the rat to develop normal carcass hexosamine levels without any dietary supply of hexosamine could not be demonstrated due to the difficulty of obtaining an hexosamine-free diet that would support good growth. All dietary protein preparations studied contain hexosamine. Rose and Fierke(11) prepared a diet containing only traces of hexosamine by the use of an amino acid mixture as the nitrogen source. They reported that the growth rate of young rats on this diet, though considerably below normal, was not improved by the addition of glucosamine. The low-hexosamine zein diet used in this study failed to support any body weight gain, although it was very similar in composition to Diet B of Kligler and Krehl(8) with which moderate growth of rats was reported. There is no apparent explanation for this discrepancy.

The hexosamine concentrations in the combined gut contents and feces were high, averaging 1% of the wet weight in Exp. III. This may be partially due to the hexosamine-rich mucins secreted by the alimentary tract(12). The intestinal flora are another possible source as indicated by the ability of certain bacteria to synthesize hexosamine(1). If such synthesis occurs within the gut, the hexosamine in the tissues of the rat could possibly be derived in part from this source.

The administration of glucosamine-HCl in the diet in an amount equivalent to that found in an adequate growth-promoting diet (.07%) had no observable effect on hexosamine levels in the carcass, urine or feces. This can be explained by postulating the destruction of the orally-administered glucosamine salt within the rat possibly by the alkaline condition within the small intestine. Destruction of free glucosamine occurs under conditions of neutral or alkaline pH(13). Naturally occurring hexosamines are less sensitive to such conditions, since they do not exist in the free state, but are incorporated into large molecular complexes such as glycoproteins, mucopolysaccharides, and nucleotides. Increased urinary output of hexosamine following oral administration of glucosamine salts to rabbits has been reported(14), however, massive doses (2 g per kg body weight) were employed.

Summary. 1. The relationship of carcass hexosamine concentration to age in growing rats showed that a marked decrease occurs between 10 and 80 days of age. 2. Hexosamine synthesis within the rat was demonstrated by means of balance studies on fasted animals and on animals fed diets containing very small or moderate amounts of hexosamine. 3. Supplementation of diets with moderate amounts of glucosamine-HCl had no effect on hexosamine levels in the carcass or excreta. 4. Hexosamines were demonstrated to occur in a number of semi-purified dietary proteins.

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Isolation of Mast Cells from Other Cellular Elements of Rat Peritoneal Fluid. (21483)

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With increasing interest developing in the physiology of the mast cell, a need has arisen for obtaining these elements in satisfactory form for quantitative studies of their chemical composition. Cell counting technics of tissue mast cells have been employed(1-3) and their numbers correlated to the concentration of certain chemical components of the tissues possessing them. To circumvent the criticisms inherent in such procedures, relatively pure mast cell populations obtained from dog mastocytomas have been examined for their heparin(4) and histamine(2) content. Question arises, however, as to whether conclusions based on mast cell tumor findings can be applied without reservation to the normal mast element.

Recently quantitative methods for mast cell enumeration in rat peritoneal fluid have been developed in our laboratories(5). In the present paper, a method is described for the separation, in this fluid, of mast cells from other cellular elements.

Materials and methods. The principle of the method involves the layering of the cell suspension, possessing relatively low density, over a high density medium. Centrifugation is employed to force the denser mast cells through the interface and these may be collected in the high density medium. Macrophages and eosinophils appear to be less dense and these are retained above the interface. Similar technics using blood albumin for separating blood leucocytes have already been reported(6). The method reported herein, however, is more rapid and can be adapted to handle larger volumes of cell suspension. To obtain peritoneal fluid, the rat is exsanguinated under ether anesthesia and the body wall incised along the mid ventral line. The cavity is then flushed with approximately 2 or 3 ml of 0.9% NaCl added in small amounts to the cavity while collecting the cells. Gentle massage of the gut through the body wall helps to dislodge more cells. The fluid from the cavity is collected with a medicine dropper. The saline (or other balanced salt solution) aids not only in obtaining more cells from the cavity but increases the fluid volume to a workable quantity and lowers the density of the peritoneal fluid. The high density medium consists of the following: sucrose (Domino brand cane sugar)—90 g; gelatin, U.S.P.—1 g; NaCl—0.45 g; and distilled water—50 ml. The gelatin is first transferred to a tightly closed bottle, moistened with some of the water and allowed to swell for about ½ hour. The sucrose and the remainder of the water are then added and the bottle placed in a 56° C incubator and shaken at frequent intervals. When dissolved, the NaCl is added and the medium is cooled. This stock solution keeps well at refrigerator temperatures. Care should be taken to prevent the inclusion of air bubbles, especially when cold. For use, each ml of stock solution is diluted with 0.4 ml of distilled water. A trace of Versene‡ may be added to this water to decrease the concentrations of ions such as calcium thus reducing the stickiness of the cells.

Separation proceeds as follows. The sucrose-gelatin solution is pipetted at the bottom of a small test tube (15 x 75 mm) to a depth of about 2 cm taking care not to touch the sides of the tubes above the expected level. The well mixed peritoneal fluid suspension is now layered carefully above the sucrose-gelatin by introducing it with a long drawn out medicine dropper and allowing it to flow against the side of the tube held in an inclined position in order to reduce the force of impact. The smaller the disturbance during layering, the

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‡ Supplies of disodium Versenate were furnished by the Bersworth Chemical Co.

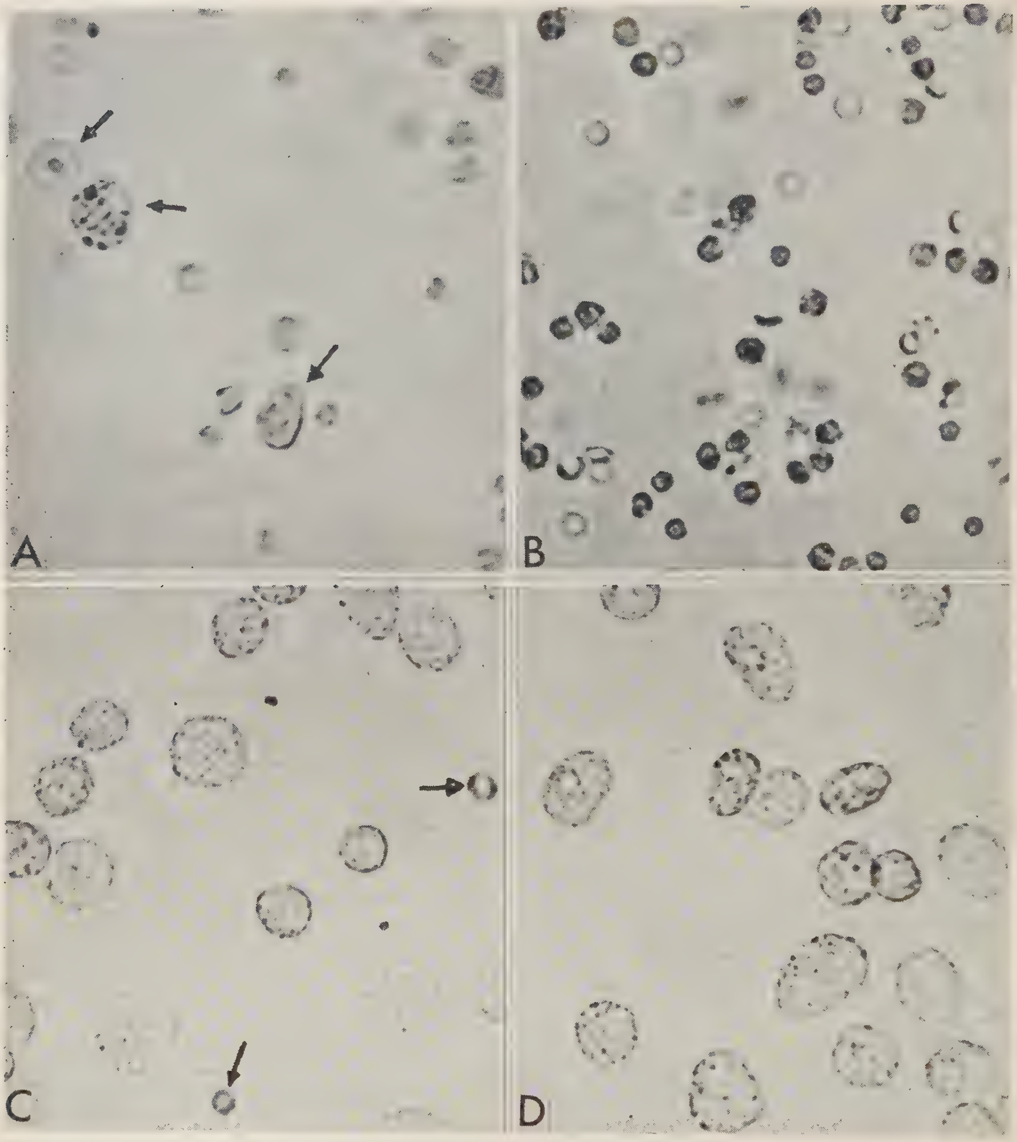

FIG. 1. Cellular elements in rat peritoneal fluid. Photomicrographs (840×) are of unfixed, unstained cells in their respective media. A. Peritoneal fluid in 0.9% NaCl before separation. Photograph taken after few hours at room temperature. Note visible nucleus in one mast cell denoting it is dead. Mast cells are marked with arrows. B. Mast-poor fraction. Small amount of peripheral blood added to peritoneal fluid to determine in which fraction erythrocytes would be retained. They are here visualized as small light cells. No mast cells present. C and D. Mast-enriched fraction. Note that mast cells are about same size as in A and that only few nuclei are visualized. Two elements other than mast cells are seen in C (arrows).

sharper the interface and the better the separation. The tube is then transferred to the centrifuge (International Centrifuge, Size 1, Type SB (1/3 H.P.) run on 110 v D.C. with the base of the tube 19.8 cm from the center of rotation) which is closed, set on the 3d graduation and allowed to run exactly 5 minutes from the time contact is made, at which point the current is cut and the cover opened, but no brake applied. When tested without load with a tachometer, the maximum speed attained (after about 1½ minutes) is 740 r.p.m. After centrifugation, the cells at the interface are collected carefully with a drawn out medicine dropper following which the high density solution is drawn up in a similar manner from the bottom with another dropper, the tip of which has been guided carefully through the interface. With proper lighting it is possible, while collecting the mast fraction, to stop the suction before contamination with interface cells occurs.

The cells thus separated can be subjected to various analyses and their biochemical reactivity delineated. The mast cells so obtained appear in good condition and react to 48-80§ in the manner described previously(7) for rat mast cells *in situ*. It is realized that some dehydration of the cell occurs due to the high osmotic activity of the sucrose solution. Although this may set some limitations on the technic, dehydration would have little import for many studies.

The various fractions obtainable are shown in Fig. 1. The entire separation procedure can be completed within 10 minutes. Rat peritoneal fluid normally possesses 3-5% mast cells. With such a starting suspension, one fraction usually contains less than 0.25% and the other more than 75% of these elements. Since mast cells are considerably larger than the other elements present, the efficiency of separation, on a total weight basis, in the mast-enriched fraction is in effect much better than the cellular counts reveal.

Summary. A method is described for the rapid separation of mast cells from other cellular components of rat peritoneal fluid. It involves differential centrifugation in a special medium which takes advantage of the greater density of the mast cell.

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§ Dr. E. J. deBeer of the Wellcome Research Laboratories kindly supplied this histamine liberator.

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Differentiation of Fibroblasts in Tissue Culture, as Determined by Mucopolysaccharide Production.* (21484)

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Among earlier theories concerning the origin of "mucin" in synovial fluid(1,2), the concept that it was derived from degenerating or damaged cells was widely discussed(3,4). Vaubel, however, reported the production of mucin clots in the medium of cultures of rabbit synovial fluid. These observations indicated that synovial "mucin" was in reality elaborated by normal proliferating cells of the synovia. These experiments have not been successfully repeated or extended to synovial tissue of other species. Vaubel also reported at that time completely negative results with media of cultures of fibroblasts derived from other tissues and stated that "with cultures of healthy fibroblasts of different origin, there occurred in no single case a precipitate of the coarse thready type, the solution remained

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clear following the addition of acetic acid or there occurred only a slight turbidity"(5).

In subsequent years, the so-called "mucin" of synovial fluid has been found to contain an acid mucopolysaccharide(6,7) identical with hyaluronic acid previously isolated from the vitreous humor and umbilical cord(8). Hyaluronic acid also was isolated from calf and pig skin(9). A substance similar to synovial fluid mucin has been found in the subcutaneous tissue of rabbits(10), in the sexual skin of monkeys, and in the connective tissue lining the astragalotibial joints of cattle(10). Therefore, it appeared possible that synovial cells might not be the only mesenchymal cells that differentiate in tissue culture and it was surmised that, if substances giving a mucin clot are produced by cells in tissue culture of synovia, similar elaboration might also occur in cultures of other mesenchymal tissues. It was thought, therefore, that this method could be utilized for determining the *in vitro* differentiation of fibroblasts derived from various types of connective tissue as well as factors influencing this differentiation.

Methods. Tissue cultures were prepared in roller tubes, in Carrel flasks, and in culture dishes(11), in an initial medium consisting of a thin layer of chicken plasma, 30% embryo extract, half diluted (EE 50), 10% horse serum (HS), and 60% amniotic fluid (AF) (12). Feeding was carried out about once a week with a solution consisting of 20% EE50, 20% HS, and 60% AF. The volume of liquid medium obtained from a single roller tube, from one Carrel flask #5, or from one to 2 Carrel flasks #3.5 was sufficient to perform the mucin clot test. At each feeding the removed liquid medium of each culture was tested separately. Following addition of 3 to 4 drops of 5% acetic acid to about 1 cc of the removed culture medium, various forms of mucin clots could be obtained, such as (a) a single large tight and ropy clump with the rest of the cloudy fluid becoming completely transparent; (b) a single small tight and ropy clump with clearance of the cloudiness in many instances; and (c) small disconnected friable thready masses and retention of the cloudy nature of the fluid. All of these were easily distinguished from diffuse precipitates and cloudiness without clot formation. One culture usually continued to give the same type of clot with each subsequent feeding. Turbidimetric determination of mucopolysaccharide was carried out by the method of Meyer and Rapport(17). To be noted is that in this method, the mucopolysaccharides are partially depolymerized by incubation with 0.02 TRU of testicular hyaluronidase[†] to prevent clot formation. The proper dilution of the fluids was determined in a preliminary run. Blanks were prepared by incubation of the proper dilution of the sample of culture medium with 5 TRU at 37°C for 30 minutes. In most experiments, the turbidities remaining after this treatment were negligible.

Results. The liquid medium from cultures of human synovia in roller tubes and Carrel flasks, from normal knees as well as from a patient with rheumatoid arthritis, gave a large mucin clot with each feeding. Addition of 5 TRU of testicular hyaluronidase[†] to the medium before acidification completely prevented mucin clot formation. Samples of the medium tested before incubation, or after incubation with various mesenchymal tissues in which growth was completely inhibited for various reasons, gave no mucin clot. The finding of Vaubel in 1933 on synovia of rabbits was thus confirmed on cultures of human synovia but contrary to Vaubel it has been found that synovia is only one of many mesenchymal tissues which in tissue cultures produce substances that give a mucin clot, the formation of which is prevented by testicular hyaluronidase.

The culture cells of the media, in which the mucin clot could be produced, were predominantly spindle-shaped and slender with fairly transparent cytoplasm, but cultures consisting only of polygonal cells with large cytoplasm filled with granules visible at low magnification also gave an equally typical large mucin clot. Contrary to the finding of Vaubel, therefore, no relationship could be found between mucin clot production, and a specific morphological feature of the fibroblasts. The mucin clot produced at each feeding was not diminished over a period of time of nearly 4 months, when feeding was discontinued. Evidence of proteolytic activity found in synovial cultures from rabbits(5) was completely absent in cultures of human synovia.

[†] Wyeth.

The supernatant solutions of cultures of subcutaneous tissue from newborn and adult rats in roller tubes, Carrel flasks, and in large bacteriological dishes regularly gave at each feeding mucin clots as typical and large as did human synovia cultures, and continued to produce this material undiminished for more than 7 months when feeding was accidentally discontinued. As soon as 12 hours after the second or third feeding, large mucin clots could be produced in the culture medium. The mucin clot could also be produced in the syneretic fluid(13) when no liquid phase was added to the plasma after coagulation with nutrient medium and the cultures were growing in the syneretic fluid of the plasma clot.

In the liquid media from cultures of human embryonic skin, mucin clots have been regularly produced. Cultures of human embryonic bone gave a mucin clot mostly of the type of disconnected thready masses. The same type of mucin clot was obtained from cultures of both membranous and long bone from young beef embryos while cultures of bone from large and older beef embryos did not give a mucin clot. Beef embryo skin gave no, or occasionally, traces of a mucin clot, evidently because epithelial growth from beef skin was predominant in these cultures. In the culture media from human fetal tendon, human fetal epiphysis, chick embryonic bone and chick embryonic heart, large tight mucin clots could be produced at each feeding. Cultures of human fetal pericardium and human fetal aorta gave mucin clots of thready masses.

These findings by themselves do not allow a conclusion concerning the extent to which differences in size and type of the mucin clot correspond to differences in amounts of mucopolysaccharides present or to differences in the physical properties of mucopolysaccharides from different types of connective tissue(15). Attempts to correlate variations of the size of mucin clots produced in media from different culture dishes with results of turbidimetric determinations of the amount of mucopolysaccharides present show that, in general, media which gave heavy mucin clots contained large amounts of mucopolysaccharides.

TABLE I. Correlation of Turbidimetrically Determined Mucopolysaccharide Concentration with Mucin Clot Production in Cultures of Adult Rat Subcutaneous Tissue, 15 Samples.

μg hyaluronic acid/ml sample	Results without enzyme	Direct method of clot production
* 96	Clotted	Large single clot
86	"	*Idem*
92	"	"
66	"	"
40	"	Small single clot
30	Turbid—no fibers	Disconnected thready masses
28	Small amt fibers	*Idem*
30	*Idem*	"
34	Turbid—no fibers	"
60	Clotted	Large single clot
146	Clotted immed.	Very large single clot
† 90	Small clot	Small single clot
80	Clotted immed.	Large single clot
93	*Idem*	Very large single clot
81	"	Large single clot

* After 2 feedings with regular medium, this culture dish was fed once with Parker's solution #866. We are indebted to Dr. R. C. Parker for his kindness in providing us with this synthetic medium.

† The same culture dish as in #1, one wk after 2nd feeding with Parker's solution #866. The length of time during which Parker's solution #866 sustains mucopolysaccharide production has not at present been determined.

In Table I, the direct method of mucin clot production described above is compared with turbidimetric determination of the amount of mucopolysaccharide produced in the culture in the time between two feedings. In column 1 are numbers of 15 Petroff culture dishes which provided samples of media, in which adult rat subcutaneous tissue had been growing. Growth in the different dishes was not uniform and the intervals between two feedings were different in different dishes. Column 2 shows the results of the turbidimetric determination of the concentration of mucopolysaccharide in the samples of the media from each dish. In column 3, results of clotting on acidification are listed, using the same technic as in column 2, but without previous treatment with 0.02 TRU of testicular hyaluronidase. In column 4 are listed the results of mucin clot production using the direct method described above. It is evident that large mucin clot production in general indicates larger amounts of mucopolysaccharides present. In all samples the turbidity disappeared or was greatly reduced by testicular hyaluronidase (5 TRU). Samples 12 to 15 were cleared by testicular hyaluronidase (5 TRU) as well as by pneumococcal hyaluronidase (5 TRU). Thus, chondroitin sulfates did not detectably contribute to the turbidity produced in the media of rat subcutaneous tissue growing in tissue culture(17).‡

The mucin clot could be produced when the percentage of EE in the culture medium was 10, 20, 30. When cultures, which gave a mucin clot in the initial medium described under methods, were subsequently fed with a medium containing 20 to 40% HS in amniotic fluid but without EE, typical mucin clots sometimes could be produced, usually only after 2 feedings with the new medium and rather irregularly. In two cases in media of cultures of adult rat subcutaneum consisting of 40% HS in amniotic fluid without EE, mucin clots have been produced for 2 months. When instead of EE, hyaluronidase-digested and filtered EE(16) in 15, 20 or 30% of the primary culture medium was used, mucin clot production was very irregular and mostly absent. Residual hyaluronidase could not be detected in the digested and filtered EE, although the possibility remains that enzyme in concentrations beyond the range of the methods used could have caused depolymerization during the long period of growth. When high concentrations of EE were used, the cells showed considerable granularity, while production of the mucin clot was at first not impaired. In concentrated EE, granularity was highest, yet the mucin clot remained at first the same or much larger. In subsequent feedings with 100% EE, however, mucin clot production soon decreased and stopped altogether. The cytoplasmic granules produced by concentrated EE were not fat granules (as shown by their equal size in a given cell, their high refractility and vital staining with neutral red), and the large mucin clot initially produced in their culture medium indicated that they were not, at least primarily, degeneration granules.

Fibroblasts in tissue culture were formerly regarded as basically non-differentiated cells and the tendency developed to designate synovial fibroblasts, in contradistinction, as "synoviablasts" which were allegedly morphologically distinguishable. The present findings, however, show that fibroblasts from various sources may differentiate and fully retain the morphological features of typical fibroblasts.

Summary. 1. Cultures of human synovia and rat subcutaneum have continued to produce, over a period of months, substances forming a characteristic mucin clot. Mucin clots have also been produced in culture media from beef embryo bone, from human embryo skin, bone, tendon, epiphysis, aorta and pericardium, and from chick embryonic bone and heart. 2. The mucopolysaccharide nature of the substances produced is attested to by the inhibition of mucin clot production by testicular and bacterial hyaluronidase. 3. Large mucin clots produced in the media of tissue cultures correspond generally to large amounts of turbidimetrically determined mucopolysaccharides produced by living cells in tissue culture. 4. Production of a mucin clot in the medium of cultures of fibroblasts from various tissues (or even a single roller tube culture) can be used as a method for preliminary orientation in determining differentiation of various types of fibroblasts, and for studying factors influencing such differentiation.

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Nature of Serum Factors Causing Agglutination of Sensitized Sheep Cells and Group A Hemolytic Streptococci.* (21485)

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Although the ability of sera from some patients with rheumatoid arthritis to agglutinate group A hemolytic streptococci and sheep erythrocytes sensitized by homologous rabbit anti-sera has been widely studied, little is known of the fundamental mechanisms involved or of the interrelationships between these agglutination reactions. Clinically, these reactions appear to be more or less specific for rheumatoid arthritis(1,11). Since any information which would aid in our understanding of the causation of this disease would be worthwhile, experiments were carried out concerning the nature of the factors causing these agglutination reactions.

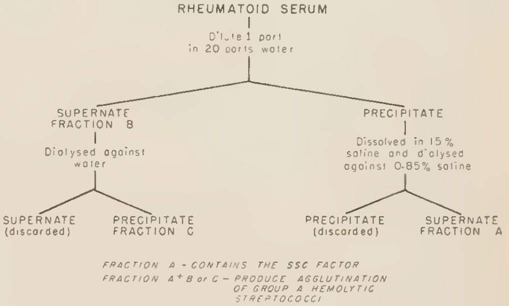
RHEUMATOID SERUM
Dilute 1 part in 20 parts water
SUPERNATE FRACTION B
Dialysed against water
SUPERNATE (discarded)
PRECIPITATE FRACTION C
PRECIPITATE
Dissolved in 15% saline and dialysed against 0.85% saline
PRECIPITATE (discarded)
SUPERNATE FRACTION A
FRACTION A - CONTAINS THE SSC FACTOR
FRACTION A+B or C - PRODUCE AGGLUTINATION OF GROUP A HEMOLYTIC STREPTOCOCCI

FIG. 1. Fractionation of rheumatoid sera by water.

Methods. 1. *Sensitized Sheep Cell* (SSC) Test: This was performed by the method of Rose *et al.*(8) modified in that one-half the agglutinating titer rather than one-half the hemolytic titer of the rabbit anti-serum was used as the sensitizing dose(7). 2. *Streptococcus Agglutination* (SA) Test. The technic of Nicholls and Stainsby(5,6) was used in which 3 strains of live group A hemolytic streptococci were employed. 3. *Serum Fractionation* by water (Fig. 1). Serum was fractionated by slowly pipetting 1 cc of serum into 20 cc of cold distilled water. The resulting mixture was allowed to stand at 4°C overnight, then centrifuged 1 hr at 2,000 rpm at 4°C. 18 sera were thus separated—16 from patients with rheumatoid arthritis and 2 normals. The precipitate was washed 3 times with 20 to 40 volumes of cold distilled water. It was then dissolved in an amount of 15% saline equivalent to its original volume and finally dialysed against 3 successive changes of 1 liter of 0.85% saline (for 5 cc of dialysate) for 3 days. The resulting precipitate was removed by centrifugation and discarded. The supernatant was retained and was designated "water insoluble" Fraction A. The supernatant resulting from the fractionation with water was lyophilized and then reconstituted to its original volume with distilled water. This was designated "water soluble" Fraction B. Fraction B was further purified by dialysing against successive changes of cold distilled water for 72 hrs. The resulting precipitate was washed and redissolved in 0.85% saline to its original volume. This was designated "water soluble" Fraction C. 4. *Absorption of SSC Factor.* It was found possible to remove all of the SSC factor from rheumatoid serum by absorption with stromata of sensitized sheep erythrocytes. For sera of high titer, it required the stromata from approximately 10 to 15 cc of packed sensitized sheep cells for each 1 cc of serum. The factor was absorbed from whole serum in 12 patients with rheumatoid arthritis. These stromata were prepared from sensitized sheep erythrocytes by alternate freezing and thawing followed by repeated washing with cold saline to remove free hemoglobin. While they were usually used immediately after being prepared, the stromata could be lyophilized without losing the power to absorb the SSC factor. Normal sheep cell (NSC) stromata could be produced by a similar method. Absorption of the SSC factor was achieved by adding 1 cc of packed stromata to the serum fraction or whole serum which had been diluted 1:4 for convenience. The mixture was incubated at 37°C for 1 hr, kept at 4°C overnight with frequent shakings, and then centrifuged at 4°C for ½ hr. To the supernatant was added further 1 cc of packed stromata and the procedure repeated. The absorption by multiple aliquots of stromata, usually 3 or 4, was found to be more efficient than a single absorption, using a large volume of stromata. It was thus possible to prepare a serum from which all detectable SSC factor had been removed without producing recognizable hemolysis. 5. *Absorption of Streptococcus Agglutination* (SA) Factor. The technic of absorption was in general similar to that described by Wager(11). Serums from 4 patients with rheumatoid arthritis were absorbed in this fashion. 6. *Paper Electrophoresis.* This was performed by the procedure described by Kunkel and Tiselius(4). Veronal buffer at pH 8.6 and a current of 600 volts, 6 amperes for 3½ to 4 hours was used. After drying, the proteins were located by the color developed by bromphenol blue followed by ammonia. Volumes of 0.005 to 0.1 cc of the test solution were used. Studies were carried out on 3 whole serums and their fractions from patients with rheumatoid arthritis.

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Results. A total of 16 rheumatoid and 2 normal sera were used in these experiments. There was a marked variation in the amount of precipitate obtained when rheumatoid serum was diluted 1:20 with water. It would appear that, in general, the 10 sera with a high SSC titer, *i.e.*, 1/1024 or greater, gave a very large amount of precipitate while the 6 with a low SSC titer, *i.e.*, 1/512 and less, and normal sera gave only a relatively slight precipitate. Among the low titer sera, there was a marked variation in the amount of precipitate obtained. As yet, none have been encountered that gave as large an amount of precipitate as the high titered sera. The "water insoluble" fraction A contained most of the SSC factor and one of the factors responsible for the agglutination of group A hemolytic streptococci. By paper electrophoresis, fraction A gives a spot only in the gamma globulin area. Absorption of this fraction with SSC stromata until all evidence of the SSC factor is removed (3 separate experiments) did not diminish the apparent size of the spot as compared to the control absorbed with NSC stromata. In one experiment the spot produced by fraction A and areas corresponding to the other serum fractions were extracted by macerating the filter paper in a small quantity of saline, 1 or 2 cc. Agglutinins against sensitized sheep

TABLE I. Relationship of the SA Factor and the SSC Factor to the Serum Fractions.

	SA titer	SSC titer
Rheumatoid serum	1/640	1/1024
Fraction A of rheumatoid serum	0	1/1024
" B *Idem*	1/40	0
" A + B "	1/640	1/1024
Normal serum	0	1/16
Fraction A of normal serum	0	0
" B *Idem*	0	0
" A + B "	0	0
Fractions A of rheumatoid serum + B of normal serum	1/640	1/1024
Fractions B of rheumatoid serum + A of normal serum	1/40	0

TABLE II. Dilution of Serum or Serum Fraction Giving a One-Plus Streptococcus Agglutination Reaction.

	Final dilution
(a) Whole R.A. serum	1/640
(b) Fraction A	1/40
B	1/640
(c) Fraction A	1/2560
B	1/40

cells, albeit of low titer, *e.g.*, ⅛ because of dilution, were obtained only from the gamma globulin area. Normal sheep cell agglutinins were not obtained, probably because of the dilution effect.

Unlike the SSC reaction, the streptococcus agglutination reaction required the presence of both fraction A and B. Table I summarizes the experiments which illustrated that while the factor in fraction A was specific for rheumatoid sera, that in fraction B was nonspecific and could be replaced by a similar factor in normal adult human serum. The greater part of this nonspecific factor in fraction B was in the globulin portion obtained by dialysis against water-fraction C. This nonspecific factor was not present in the serum from sheep, rabbit or guinea pig. *Experiments with the factors in the streptococcus agglutination system.* An attempt was made to determine which of the 2 components of the streptococcus agglutination system determined the level of the streptococcus agglutination of the whole sera. In 2 experiments, using the same serum and its fractions, the following titrations were carried out with appropriate controls: (a) Whole serum. (b) The fraction A was held constant at a final dilution of 1/40 in each tube. The fraction B was in doubling final dilutions from 1/20 to 1/2560. (c) The fraction A was in doubling final dilutions from 1/20 to 1/2560. The fraction B was held constant at a final dilution of 1/40 in each tube. The results are shown in Table II. It is thus seen that in this one serum, at least, the height of the agglutination titer depended upon the nonspecific factor B and that, if more had been present, the SA titer would have been much greater.

The ability of rheumatoid serum to agglutinate streptococci may be diminished or removed by absorption with streptococci(11). The relationship of the two components of the streptococcal agglutination system to this phenomenon was investigated in one experiment using fractions of a rheumatoid serum of high SA titer.

The 2 fractions were absorbed separately with streptococci. The absorbed fraction was then combined with the opposite nonabsorbed fraction and the ability to agglutinate streptococci was observed. The results showed that the nonspecific factor in fraction B was readily removed by absorption. There was no diminution of the titer of the system when fraction A was absorbed with streptococci.

A further experiment (Table III) was performed in which an unfractionated serum with a high streptococcus agglutination titer was absorbed with streptococci. After absorption the serum was mixed with the nonspecific factor of fraction B from a normal adult serum, and the SA titer was determined. While there was a diminution in the SA titer, there was not a complete disappearance. The control in which the absorbed serum was used alone showed no agglutination for streptococci. These experiments would indicate that while the nonspecific factor is readily removed by streptococci, the specific factor is unaffected when absorbed with streptococci alone and only moderately absorbed by streptococci in the presence of the nonspecific factor.

Relationship between the SSC and SA reactions. The experiments outlined in Table III confirm the observations that the SSC titer is not affected by absorption of rheumatoid sera

TABLE III. Relationship between SSC Factor and Streptococcus Agglutinating (SA) Factor.

Serum or serum fraction	SA titer	SSC titer
Rh.s.*	1/1280	1/2048
" + n.f. B	1/1280	1/2048
" absorbed with str.	0	1/1024
Idem + n.f. B	1/320	1/1024
Rh.f. A	0	1/1024
" + n.f. B	1/640	1/1024
" absorbed with SSC stroma + n.f. B	1/80	0

* Rh.s. = rheumatoid serum; n.f. = normal fraction; str. = streptococci; Rh.f. = rheumatoid fraction.

with streptococci. The small drop in titer for sensitized sheep cells in the streptococci-absorbed serum was also observed in the temperature and dilution controls. The complete absorption of the SSC factor from serum by sensitized stromata caused a fall but not a complete disappearance of the streptococcus agglutinating titer. These changes may be illustrated by the results in one serum in which the SA titer fell from 1/160 before absorption to 1/80 after absorption. In another serum, the fall was much greater being from 1/640 before absorption to 1/80 after absorption. In this same serum, absorbed with an equivalent amount of normal stromata, the titer fell to 1/320. The SSC factor was removed from a sample of fraction A from rheumatoid serum by absorption with SSC stromata. This absorbed fraction was then mixed with the fraction B of normal serum. The results (Table III) would indicate that the streptococcus agglutination reaction was only minimally affected by this procedure. It would seem from these experiments that 2 distinct but closely related phenomena account for the ability of rheumatoid serum to agglutinate streptococci and sensitized sheep cells.

Discussion. In agreement with previous workers(8,9,10,12), the factor in serum from patients with rheumatoid arthritis responsible for the agglutination of sensitized sheep cells was found to be in the gamma globulin fraction of serum precipitated by dilution with water. Two components, however, are necessary for the streptococcus agglutination system. One of these is specific for rheumatoid serum, is a gamma globulin or is closely associated with these globulins, and is related to but not identical with the SSC factor as determined by absorption experiments. The second component, soluble in water, was found as well in normal human adult serum. It has not as yet been demonstrated to be an antibody. It would seem that the first part of the streptococcus agglutination reaction is probably the union between the streptococci and the nonspecific factor. Following this combination, the specific component can complete the reaction and cause agglutination of the streptococci.

It is not known whether the gamma globulins responsible for the SSC and SA reactions represent merely an increase in normal components or the appearance of abnormal elements. It may be that the precipitate reflects changes in the relative amounts of groups of water soluble proteins as was first shown by Kendall(2,3).

Summary. 1. The SSC factor was demonstrated to be in the gamma globulin fraction of rheumatoid serum precipitable by dilution with water. 2. The SA reaction required the presence of both the water insoluble fraction and supernate. The former was specific for rheumatoid serum, the latter was found to be a component of normal adult human serum. 3. Reciprocal absorption experiments would indicate that the SSC reaction and the SA reaction are due to separate constituents of the gamma globulin.

I would like to thank Dr. Harry M. Rose for his advice.

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Comparative Studies on Toxins of *Pasteurella pestis* and *Pasteurella pseudotuberculosis.* (21486)

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Opinions of different writers in regard to the nature of plague and pseudotuberculosis toxins vary considerably. As summarized by Pollitzer (1) most investigators classify the toxin of *Pasteurella pestis* as an endotoxin. Girard *et al.*(2) pointed out in this connection that the plague toxin lacks the glucolipid complex found in numerous other Gram-negative bacteria. On the other hand, according to Ramon *et al.*(3), the thermolability of this toxin and its transmutation into toxoid represent features usually met with in exotoxins. The toxin of *Pasteurella pseudotuberculosis* has also been classed as an endotoxin(4,5), but recent investigations by Thal(6) showed that certain strains of this species can produce toxic substances immunologically resembling true exotoxins.

In view of these discrepancies it seemed desirable to study the pathophysiological action and the immunological relationship of the two above-discussed toxins.

Materials. Plague toxin. With the methods of Englesberg and Levy(7), plague toxin was produced by growing the avirulent strain E.V. 76 (Girard and Robic) in a casein-hydrolysate-mineral-glucose medium at 28°C for 7 days and precipitating the toxic substance from the supernatant fluid by 100% saturation with ammonium sulfate. The precipitate was suspended in 35% saturated ammonium sulfate solution, the insoluble part removed by centrifugation and the toxin reprecipitated by addition of ammonium sulfate to 70% saturation. Ammonium sulfate was then removed by dialysis against distilled water. The plague toxin isolated with the aid of this method, though partially purified, was still electrophoretically inhomogenous. *Pseudotuberculosis toxin*: Strain 105/III of *P. pseudotuberculosis* was grown for 2 days at 37°C on Huntoon's modified hormone agar in Roux bottles. Approximately 50 billion organisms, harvested and washed with physiological saline solution, were suspended in 100 ml of an M/150 phosphate buffer (pH 7.5) and killed by addition of 0.5 ml toluene and incubation for 24 hours at 37°C. Extraction of the toxin was completed by addition of 1% sodium bicarbonate to the suspension. The insoluble bacillary residue—being of negligible toxicity—was removed by centrifugation and discarded. The supernatant was dialyzed against distilled water for 24 hours in the refrigerator. The product thus obtained, which contained 7 mg dry material per ml, will in the following be called the "toxic extract." For comparison, extracts obtained by the same method from nontoxic, but virulent, strains of *P. pseudotuberculosis* (14/I and 16/II) were used. *Antitoxic plague serum* was produced by immunization of rabbits with the partially purified plague toxin over a period of 4 months. Pseudotuberculosis antitoxic serum was prepared by immunizing rabbits with toxoid and the toxin of strain 43/III (6). *Princeton mice* (average weight 20 g), white rats (average weight 250 g) and guinea pigs (300 to 400 g) were the test animals.

Results. Lethality of toxins for certain laboratory animals. The median lethal doses(8) for mice and rats—expressed in μg per animal—are given in Table I. As previously established by Thal(6), rabbits (approximately 2 kg) are highly susceptible to the pseudotuberculosis toxin, succumbing invariably to 2 mouse LD_{50} given intravenously. However, according to Schar and Meyer(9) these ani-

TABLE I. LD_{50} of Plague and Pseudotuberculosis Toxin for Mice and Rats.

	LD_{50}, μg/animal	
Toxin	Princeton mouse 20 g	Albino rat 200 g
P. pestis	.2	2.6
P. pseudotuberculosis	22	230

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* Guest investigator from the State Veterinary Medical Institute, Stockholm, Sweden.

mals are very resistant to the plague toxin, an amount equivalent to 300,000 mouse LD$_{50}$ (60 mg), administered intravenously, not producing a lethal effect. High resistance against the plague toxin was also observed in guinea pigs. However, this animal could be killed invariably with 1 mg of the toxic extract (50 mouse LD$_{50}$) of *P. pseudotuberculosis*. In Thal's studies, out of 186 *P. pseudotuberculosis* strains only 8, all belonging to serologic group III, produced toxins in 48-hour broth cultures, while the others failed to do so even if subjected to autolysis for 6 months. Extracts prepared from toxic and nontoxic strains with trichloracetic acid(10) or diethylene glycol(11) proved nontoxic if administered to mice in 1 mg doses. Nor could toxic substances be found in the supernatant of casein hydrolysate-mineral-glucose cultures of *P. pseudotuberculosis* incubated for 6 days. As noted, this contrasts sharply to the behavior of *P. pestis*.

Pathophysiologic effects of the toxins. In mice and rats receiving large doses (5 or more LD$_{50}$) of the plague toxin intravenously hemoconcentration due to impaired function of the peripheral vascular system always became marked within 2 to 4 hours(9). In rats, blood pressure fell gradually to shock levels and this fall was accompanied by hemoconcentration with an increase of over 25% in the number of erythrocytes per cmm. The animals died always within 4 hours after intravenous administration of a large toxin dose. After a small dose (*e.g.* 1 LD$_{50}$) of plague toxin, however, hemoconcentration was not significant, and about 50% of the animals died within 4 to 24 hours and a very few thereafter. In rats and mice which did not succumb within 10 hours, the liver function, revealed in retention of bromsulfalein and also in bilirubinemia and jaundice, was disturbed(9). Response of rats and mice to subcutaneously inoculated plague toxin consisted of extensive local edema followed by skin necrosis and scar formation.

Post mortem findings. In animals which died within a few hours after toxin administration, hyperemia of subcutaneous tissue and clear colorless or slightly yellowish exudates in the peritoneal and sometimes also in the pleural cavities, were found. In rats frequent evidence of hemorrhages was seen in the small intestines. Animals sacrificed or succumbing one day after toxin was inoculated showed, in more than 50% of the cases, fatty degeneration and multiple disseminated necrosis of the liver. Kidneys revealed various degrees of parenchymatous degeneration.

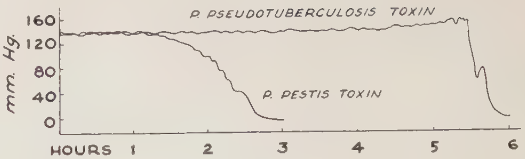

FIG. 1. Arterial blood pressure in rats.

Toxin of *P. pseudotuberculosis* also caused skin necrosis in mice and rats when the toxin was subcutaneously or intracutaneously administered. In rats receiving the pseudotuberculosis toxin intravenously, pathological changes in the liver were identical to those seen after plague toxin. However, the behavior of the blood pressure of rats given a large dose of pseudotuberculosis toxin intravenously (5 or more LD$_{50}$) was *strikingly different*. Blood pressure remained on normal level for 5 to 6 hours; then respiration which had been irregular for about one hour ceased, and blood pressure dropped to zero. The trend of blood pressure of rats after administration of pseudotuberculosis toxin and plague toxin is illustrated in Fig. 1. Pseudotuberculosis toxin did not cause significant hemoconcentration in mice or rats. The average erythrocyte count of 10 mice 4 hours after intravenous administration of 10 LD$_{50}$ of toxin was 11,100,000 per cm, as compared to 10,700,000 in 10 control mice of the same age and weight.

Specific neutralization. 1. *In vitro* neutralization of pseudotuberculosis and plague toxin by antitoxic sera: Various amounts of toxin were incubated at room temperature for 3 hours with 2 standard concentrations of antitoxic sera. Each of 4 mice in one group received 0.5 ml of a serum-toxin mixture intravenously. In Table II results, in terms of number of mice dying within 48 hours, are given. 2. *In vivo* neutralization tests: Sera were injected intraperitoneally 20 hours before toxins were administered intravenously. The results are recorded in Table III. Plague

TABLE II. *In Vitro* Neutralization of Pseudotuberculosis and Plague Toxin by Antitoxic Sera.

Toxin		No. mice dead 48 hr after injection*				
		Pseudotuberculosis antitoxic serum		Normal rabbit serum	Plague antitoxic serum	
		Amt/.5 ml inoculum, ml				
Type	Dilution	.005	.025	.025	.025	.10
Pseudotuberculosis	1:5	4	4	4	4	4
	1:10	4	3	4	4	4
	1:20	4	0	4	4	4
	1:40	4	0	4	4	4
	1:80	0	0	4	4	4
	1:160	0	0	4	4	4
	1:320	0	0	2	3	2
	1:640	0	0	0	0	0
LD_{50}		1:56	1:12.5	1:320	1:400	1:320
NI†		5.7	25.6	1.0	.8	1.0
Plague	1:500		4	4	4	4
	1:1000		4	3	2	1
	1:2000		1	1	0	0
	1:4000		0	0	0	0
LD_{50}			1:1600	1:1400	1:1000	1:800
NI†			.9	1.0	1.4	1.7

* Toxins incubated with sera 1 hr at 37°C previous to injection. 4 mice/group received 0.5 ml of serum-toxin mixture intravenously.

† Neutralization index is the LD_{50} of toxin incorporated in antitoxic serum (AS) over the LD_{50} of toxin incorporated in normal serum (NS). $\text{N.I.} = \frac{LD_{50}\,(AS)}{LD_{50}\,(NS)}$.

toxin afforded no protection at all when the serum was given in advance of the toxin, neutralization indices never being significantly different from 1.

As will be observed from Tables II and III, the immunobiological behavior of the 2 toxins differed significantly. The pseudotuberculosis toxin was neutralized by its antitoxic serum in multiple proportions whether given simultaneously with the serum or subsequently. Plague toxin was neutralized to a small extent and only when administered after *in vitro* incubation with its antitoxic serum. Furthermore it may be observed from Table II that no cross immunity exists between the 2 toxins and their antitoxic sera.

TABLE III. *In Vivo* Neutralization of the Toxin of *P. pseudotuberculosis* by Homologous Antitoxic Serum.

	No. mice dead 48 hr after inj.*			
	Pseudotuberculosis antitoxic serum			Normal rabbit serum
Toxin dilution	Amt given 20 hr before toxin, ml			
	.01	.05	.25	.25
1:10	4	4	1	4
1:20	4	3	0	4
1:40	4	2	0	4
1:80	3	0	0	4
1:160	2	0	0	3
1:320	0	0	0	1
1:640	0	0	0	0
LD_{50}	1:140	1:34	1:8	1:230
NI†	1.6	6.7	28	1.0

* Sera administered intraperitoneally. 4 mice/group inj. intravenously with 0.5 ml toxin.

† Neutralization index is the LD_{50} of toxin incorporated in antitoxic serum (AS) over the LD_{50} of toxin incorporated in normal serum (NS). $\text{N.I.} = \frac{LD_{50}\,(AS)}{LD_{50}\,(NS)}$.

Skin tests. Intracutaneous administration of both toxins to rats caused local, hemorrhagic, necrotic lesions. In guinea pigs only the toxin of pseudotuberculosis caused such lesions. When the toxins were mixed with homologous antitoxic sera and given intracutaneously the effect of pseudotuberculosis toxin was neutralized, but the effect of the plague toxin was not.

Discussion. Toxins of plague and pseudotuberculosis share certain physicochemical properties. Both are thermolabile (destroyed at 60°C within one hour) and easily converted into toxoid by formaldehyde.

With regard to toxicity, their pathophysiologic action and their immunologic behavior, the 2 toxins differ, however, strikingly. The pseudotuberculosis toxin was highly lethal for rabbits, rats and mice, to a lesser degree also for guinea pigs. Though the plague toxin is endowed with a high toxicity for mice and rats, even in amounts of 300,000 mouse LD_{50} it never exerts a lethal effect on rabbits and guinea pigs.

While the toxin of *P. pestis* caused marked hemoconcentration and gradual fall of blood pressure in rats, this typical trend did not follow injection of pseudotuberculosis toxin. Equally striking are the immunological differences. In the case of *P. pseudotuberculosis* toxin, active immunity against multiple lethal doses could be produced in rabbits, and neutralization of the toxin *in vivo* and *in vitro* in multiple proportions was possible. The serum of rabbits immunized with plague toxin did not protect mice against more than 3 mouse LD_{50} given intravenously. These immunological discrepancies are also reflected in the results of skin tests performed on rats and guinea pigs. Thus the toxin of certain strains of *P. pseudotuberculosis* fulfills all the requirements of a true exotoxin as defined by Topley and Wilson(12) and Doerr(13). The immunological properties of the plague toxin, on the other hand, did not conform to these requirements. According to the now prevailing opinion, the endotoxin in Gram-negative bacteria is identical with somatic O antigen (14,15). If the chemical structure of *P. pseudotuberculosis* conforms to this conception, this species ought to have 2 toxins—the exotoxin investigated by us and an endotoxin hitherto not satisfactorily defined.

Summary. 1. Mice and rats were highly susceptible to the toxin of *P. pestis* and of *P. pseudotuberculosis;* rabbits and guinea pigs were refractory to the plague toxin but succumbed to the toxin of *P. pseudotuberculosis.* 2. Plague toxin caused hemoconcentration and a gradual fall of the blood pressure in rats. These changes are not demonstrable after administration of pseudotuberculosis toxin. 3. The pseudotuberculosis toxin could be neutralized by its antitoxic serum *in vivo* and *in vitro* in multiple proportions, thus showing it to be a true exotoxin, whereas the plague toxin could be neutralized only to a negligible extent. 4. No cross immunity to these 2 toxins was evident.

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Response of Liver Enzymes and Other Proteins to Amino Acid Deficient Diets.* (21487)

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In recent years many workers have demonstrated sensitivity of various tissue enzymes to dietary changes(1-5). This sensitivity has led to studies in this laboratory concerning effect of single amino acid deficiencies on rat liver enzyme systems(6-9). These findings obtained in the previous studies in this laboratory(6-9) have been extended in the present work in an attempt to explain why different patterns of activity are obtained with different amino acids. To this end, we have studied the effect of first depleting animals of liver enzymes by feeding a non-protein ration and then feeding back incomplete amino acid mixes as the source of protein in the rations. The liver enzyme systems chosen for the present study include xanthine oxidase, a soluble cytoplasmic enzyme; succinic oxidase and choline oxidase, mitochondrial systems; and endogenous respiration, the resultant activity of oxidative enzymes acting on endogenous substrates, which should be a measure of general liver metabolic rate. In addition, we have studied the changes in liver nitrogen *per se*.

Methods. Weanling male albino rats (Holtzman Rat Co., Madison) were used as experimental animals in these studies. They were first fed a non-protein synthetic ration consisting of Salts IV (4%)(10), corn oil (5%), the known vitamins in sucrose (2%), and sucrose to make 100%. All animals were individually housed in screen-bottomed cages and offered food and water *ad libitum*. After 9 days on the non-protein ration, the animals were divided into 4 groups of 8 animals each. Group I was fed the purified ration of Ramasarma, Henderson and Elvehjem(11) consisting of a complete purified amino acid mix (16%), Salts IV (4%)(10), corn oil (5%), the known vitamins (2%), and sucrose to make 100%. Group II was fed the complete ration minus lysine; group III, the complete ration minus histidine; and group IV, the complete ration minus methionine. After 8 days on these purified amino acid diets, the animals were stunned by a blow on the head, decapitated, and the livers placed immediately in cracked ice. A portion of each liver was weighed, homogenized in 5 volumes of 0.039M sodium potassium phosphate buffer (pH 7.3), and strained through gauze. These homogenates were used for xanthine oxidase determinations according to the method of Axelrod and Elvehjem(12) and choline oxidase by the method of Williams, *et al.*(13). A portion of the homogenate was diluted with buffer to give a 5% homogenate which was employed for measuring succinic oxidase according to the method of Schneider and Potter(14). Endogenous respiration was calculated from the oxygen uptake of the control flasks in the xanthine oxidase assay during the first 10-minute interval following the temperature equilibration period. All enzymes were assayed at 37° with a Warburg bath. Nitrogen of the homogenates was determined by a semi-micro Kjeldahl procedure in duplicate. For additional controls the above enzymatic studies were also carried out on weanling animals as received from the rat company to obtain zero time activity, on animals fed the non-protein ration for 9 days, and on animals fed the complete purified ration of Ramasarma *et al.*(11) for the entire 17 days of the experiment.

Results. The initial 9-day non-protein regimen produced a 10% decrease in body weight of the weanling rats employed. Subsequent feeding of single amino acid-deficient diets led to a further slight weight decline, (Groups II-IV) while supplementation with the complete amino acid mixture gave a prompt increase in body weight of over 50% after 8 days (Group I). There was no significant variation in food consumption among the groups of rats supple-

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† Captain in U.S.A.F. (M.S.); sponsored by U.S.A.F. Institute of Technology.

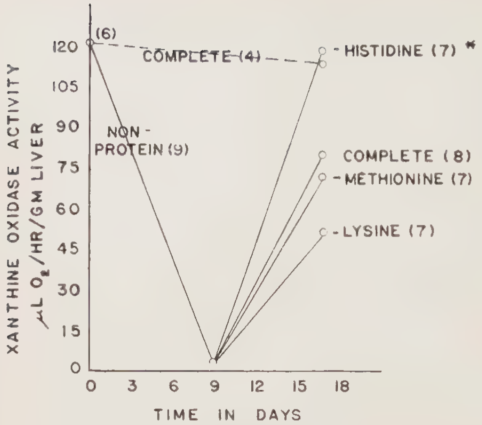

FIG. 1. Response of rat liver xanthine oxidase to amino acid-deficient diets.

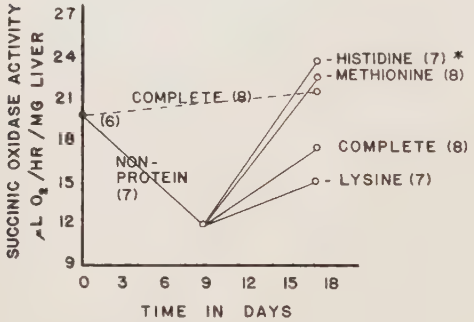

FIG. 2. Response of rat liver succinic oxidase to amino acid-deficient diets.

mented with the various amino acid-deficient diets.

The results of the enzyme studies and liver nitrogen determinations are presented in Fig. 1-4. The results have been calculated in terms of fresh liver weight since in these experiments we are primarily interested in actual synthesis of these enzymes and not necessarily whether the enzymes are synthesized at a faster or slower rate than other liver nitrogen. A separate curve is presented for the corresponding changes in concentration of liver nitrogen under these experimental conditions (Fig. 4) and an estimation can be made of the relative rates of change of the liver enzymes with liver nitrogen. As shown in Fig. 1, feeding of a complete synthetic diet minus histidine resulted in a return of xanthine oxidase activity to a level held by pre-experimental controls and maintained for 17 days by animals subsisting on a complete synthetic diet. Also shown in Fig. 1 is the finding that rats fed a non-protein ration for 9 days followed by a complete synthetic diet minus lysine for 8 days gave a xanthine oxidase response similar to that observed when animals were given a diet deficient in lysine without a prior protein-depletion period(9). The result in either case was a xanthine oxidase activity averaging about 50% of normal.

Supplementation with a methionine-free diet, however, gave xanthine oxidase activity results which were somewhat different from those obtained when a methionine-free ration was fed without a prior protein-depletion period(6). Under the conditions of the present experiment, a significant increase in level of activity was observed (Fig. 1), whereas in animals fed a methionine-free ration from the beginning(6) there was a total removal of xanthine oxidase activity.

An additional point of interest in the xanthine oxidase responses recorded in Fig. 1 is the finding that this activity was not completely restored after the addition of a complete synthetic diet. It is possible that weanling rats preferentially employ the amino acids from the complete ration for growth, and

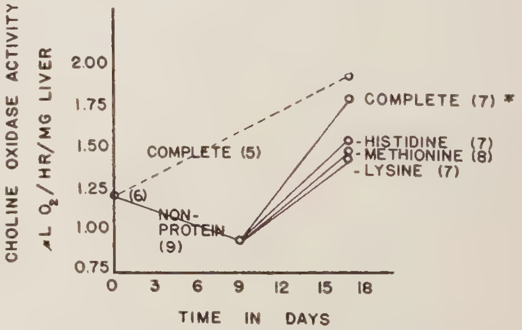

FIG. 3. Response of rat liver choline oxidase to amino acid-deficient diets.

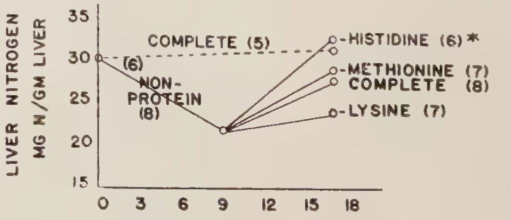

FIG. 4. Effect of amino acid-deficient diets on rat liver nitrogen concentration.

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* Figures in parentheses in Fig. 1-4 indicate No. of animals used to obtain avg value for the points.

thus xanthine oxidase synthesis may be limited to the extent observed.

For the mitochondrial enzymes, succinic oxidase and choline oxidase, results generally similar to those for xanthine oxidase were obtained. These results are presented in Fig. 2 and 3. The non-protein ration decreased the activity of these enzymes to a smaller extent than in the case of xanthine oxidase. However, the order of effectiveness of the histidine-, methionine-, and lysine-deficient rations in returning the activity of succinic oxidase and choline oxidase was essentially the same as for xanthine oxidase. With choline oxidase an additional effect was observed in that the enzyme activity increased with the age of the animals so that all of the curves were skewed upward with time.

No significant change in endogenous respiration was noted throughout the experiment. Therefore, a figure for these results is not presented.

As shown in Fig. 4, there was a drop in liver nitrogen in the animals fed the non-protein ration. When the animals were supplemented with the histidine- and methionine-deficient rations, the redeposition of liver nitrogen occurred at an equal (methionine) or faster (histidine) rate than when the rats were supplemented with the complete ration. The lysine-deficient ration gave a significantly lower liver nitrogen concentration than any of the other groups, however.

Discussion. A single, general explanation for the findings reported in this paper is difficult to set forth. However, since the activity of the liver enzymes and liver nitrogen concentration increase when the amino acid-deficient rations are fed to protein-depleted animals, some "utilization" of the incomplete proteins must take place. The missing amino acids may be salvaged from the body proteins to an extent sufficient to allow some synthesis of liver enzymes and other proteins. It is interesting that the least return to normal of all the variables studied was obtained with the lysine-deficient ration. This observation fits with the possibility that lysine should be one of the most limiting amino acids in protein-depleted animals, since once lysine has been deaminated, it cannot be reaminated. Thus the extent of salvage of this amino acid from body amino acid pools would be severely limited.

In general the results of this paper correlate well with studies reported earlier concerning the effect of individual amino acid deficiencies on liver enzyme activities(6,8,9). In the earlier studies, however, the animals were not depleted of body protein before the amino acid-deficient rations were fed. One discrepancy between the present and earlier studies is that a methionine-deficient ration fed to rats not previously depleted of protein produced a total loss in liver xanthine oxidase activity. In the present studies the feeding of a methionine-deficient ration to protein-depleted rats produced a measurable rise in liver xanthine oxidase activity. It is interesting to note, however, that in the earlier histidine(8) and lysine(9) deficiency studies liver enzymes were lost to a much smaller extent than would be expected if none of the incomplete dietary proteins were utilized. In the present studies the liver enzymes and other liver protein actually increased when protein-depleted rats were fed the same amino acid-deficient rations. Therefore, the two types of studies tend to support each other to the extent that rations deficient in certain essential amino acids can be utilized to some extent in building or maintaining liver proteins.

Summary. 1. Histidine-deficient diet can completely restore xanthine oxidase activity to livers of rats previously depleted of such activity by a non-protein diet; methionine-free and lysine-free diets partially restore such activity with the lysine-free diet the least active in this respect. 2. Liver succinic oxidase activity can be similarly restored by histidine- and methionine-free rations but only partially restored by a lysine-free ration. 3. Liver choline oxidase activity of protein-depleted rats can be restored partially and to about the same extent by the 3 amino acid-deficient diets studied. 4. Liver nitrogen concentration follows approximately the same pattern as succinic oxidase in these studies. 5. The enzymatic responses to a non-protein diet followed by single amino acid-deficient diets have been compared to responses observed in earlier

studies under conditions of initial single amino acid-deficient diets.

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Urinary Bladder Response to Unilateral Stimulation of Pelvic Nerves.* (21488)

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It is well known that motor responses of the urinary bladder can be elicited by stimulating appropriate components of either division of the autonomic nervous system(1-4). This presents a disturbing problem to the functional anatomist who is accustomed to thinking of a normal counteraction of the effector mechanisms of an organ so innervated. Some investigators feel that parasympathetic and sympathetic nervous stimulation of the bladder produce responses that differ only in magnitude or degree(5-8). While this view is not shared by the majority, it remains to be shown in what respects the responses are similar or dissimilar. It was found in our laboratory that bilateral excitation of hypogastric nerves evokes a diphasic response of all portions of the detrusor muscle; also, that unilateral excitation elicits a prompt contraction followed by relaxation on the corresponding side and usually a similar, though less extensive response on the contralateral side.

In the present paper we are reporting the results of a similar study on the pelvic nerves. As in the previous studies(1,2) we have employed a photographic technic which makes it possible to record simultaneously the activity taking place in various portions of the bladder during a given stimulus.

Methods. Cats were used. Anesthesia was obtained by administration (i.p.) of 45 mg chloralosane and 25 mg Dial (Ciba) per kg body weight. Following a midventral abdominal incision the bladder was oriented so that either the ventral or dorsal surface was uppermost. On this surface were placed small squares of white adhesive paper, each bearing central spot of black ink. The paper squares were arranged so as to divide the exposed surface into quadrants (A,B,C,D) and 12 segments(1,2). By means of a Ciné-kodak Special 16 mm camera mounted above the bladder, photographs were taken at one-sec-

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Figs. 1 and 2 are outline sketches of bladder showing various regions, A1 through D2, in which local activity of muscle was observed in response to unilateral stimulation of pelvic nerves. A graph of response in each of 12 segments has been placed on this sketch as near as possible to the region from which the observation was drawn. Each graph represents a complete observation from a single segment. Each circle in each graph represents activity during each second of observation. Onset of stimulation coincides with first circle in each graph; cessation of stimulation indicated by arrow. Each figure contains 2 complete experiments: A (open circles, interrupted lines), and B (closed circles, continuous lines). Right half of each figure, represented by segments B3, C3, A2, B4, C4, D2, indicates homolateral side of bladder wall while left half of figure, represented by other 6 segments, indicates contralateral half of bladder wall. Insert: kymograph record of bladder volume during each period of stimulation.

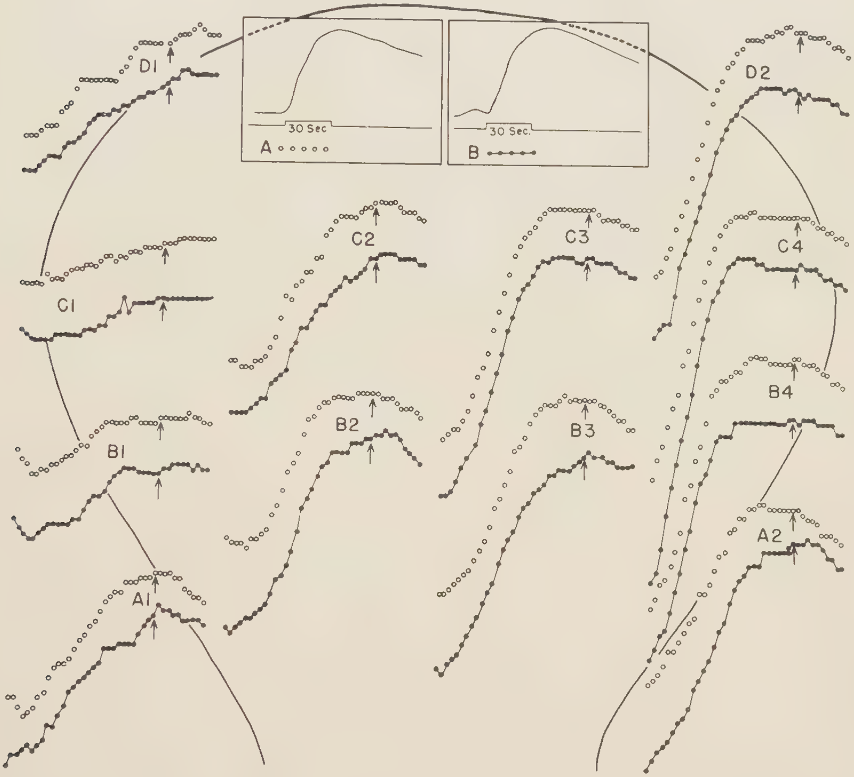

FIG. 1. Bladder responses in Cat 9 recorded at intravesical pressure of 11 cm water and with hypogastric nerves intact. A (Exp. 6, open circles, interrupted lines), unilateral stimulation of left pelvic nerves with all pelvic nerves intact. B (Exp. 8, closed circles, continuous lines), unilateral stimulation of distal cut ends of left pelvic nerves with opposite pelvic nerves intact.

ond intervals during the time of the experiment. By projecting the film record onto suitable paper, measurements were made of the activity per unit length of muscle in each of the 12 segments during each second of time. The results were plotted to show a graph of the response in each case. Minimal strength, square wave stimuli were used (Model S4A Grass stimulator) at a frequency of 30/second and a 5 millisecond duration. Kymographic records of volume changes under conditions of constant intravesical pressure were made in all experiments(1).

Results. Unilateral stimulation of intact pelvic nerves usually gave an immediate, vigorous and sustained contraction of the detrusor muscle on the corresponding side with a slightly weaker and often somewhat delayed response on the opposite side (Exp. A, Figs. 1 and 2). Occasionally the contraction on the contralateral side would be preceded by relaxation (Figs. 1A, segments A1, B1, and 2A,

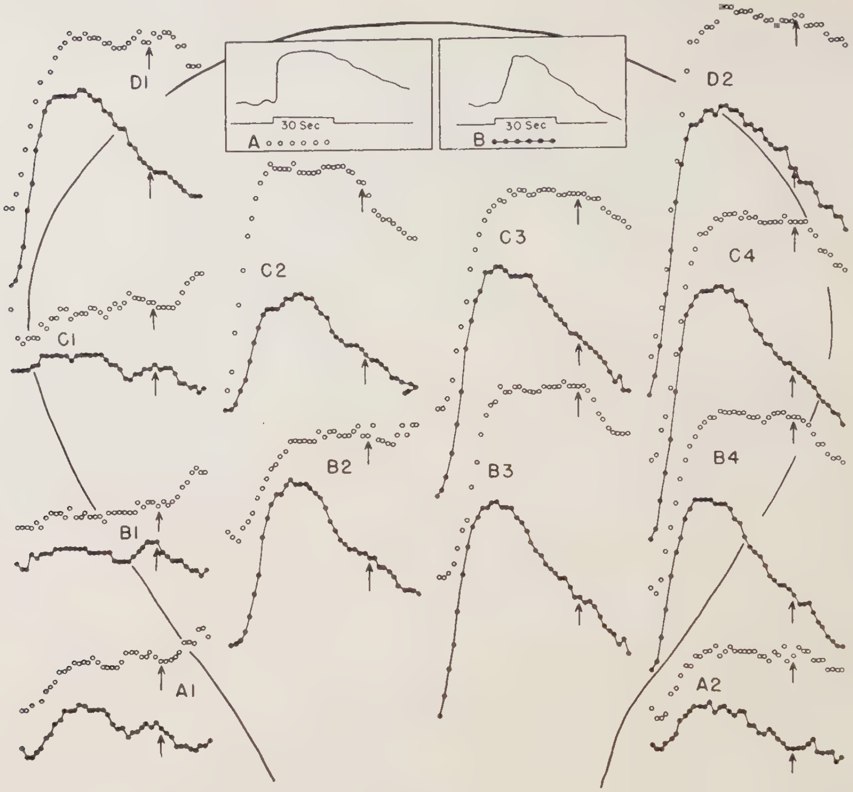

FIG. 2. Bladder responses in Cat 3 recorded at intravesical pressure of 13 cm water and with hypogastric nerves intact. A (Exp. 1, open circles, interrupted lines), unilateral stimulation of right pelvic nerves with all nerves intact. B (Exp. 3, closed circles, continuous lines), unilateral stimulation of distal cut ends of right pelvic nerves with opposite pelvic nerves intact.

segment B2) or the relaxation would continue throughout the period of stimulation.

Section of the pelvic nerves on one side and stimulation of their distal cut ends did not alter the response appreciably (compare experiments A and B of Fig. 1). Section of all of the pelvic nerves with excitation of their distal cut ends on one side evoked responses which were strikingly similar in most respects to those obtained from the same nerves before section. In some of these studies the average amount of shortening per unit length of muscle on the contralateral side was actually greater following section of the nerves than in the intact bladder. Section of the hypogastric nerves did not alter the general pattern of the response when intact or sectioned pelvic nerves were stimulated.

A few investigators have reported results similar to ours following unilateral stimulation of intact pelvic nerves(8-10) but they ascribed the responses obtained on the contralateral side to central reflex mechanisms since they found that after sectioning the nerves, stimulation of their peripheral cut ends limited the contraction almost exclusively to the stimulated side. However, most workers(7,11-14) noted an almost completely one-sided contraction in response to unilateral stimulation of intact pelvic nerves. Debaisieux(5) has been one of the few who insisted that there was no essential difference in the bladder response from stimulation of intact nerves as opposed to excitation of their distal cut ends.

An unusual type of response was noted in two studies. In one, all regions of the ventral surface of the viscus contracted immediately and with great vigor during unilateral stimulation of intact pelvic nerves but within a few seconds the majority of the bladder segments relaxed in much the same manner as during hypogastric stimulation(2). This response did not recur when the posterior surface was studied nor when the opposite pelvic nerves were excited. In the other observation (Exp. B, Fig. 2), unilateral stimulation of the distal cut ends of the divided pelvic nerves evoked a similar reaction. Stimulation of these same nerves previous to section had yielded the usual parasympathetic response (Exp. A, Fig. 2). The unsustained responses were elicited at the same intravesical pressures and with the same parameters of stimulation as were employed in all the other experiments. The unsustained responses differed from those evoked by hypogastric stimulation in that some segments of the bladder continued to contract during stimulation of the pelvic nerves. This was never the case during hypogastric stimulation. Others(8,15,16) have reported similar bladder responses which were attributed, among other causes, to high or low intravesical pressures or to spinal cord reflex mechanisms.

Section of the pelvic nerves on one side and stimulation of their central cut ends evoked, after a latent period of several seconds, a well marked contraction on both sides of the bladder. Section of the opposite pelvic nerves was found to abolish this response even though the hypogastric nerves were intact. Others (5,9,11) have reported similar results except that the contraction was confined to the side of the bladder which had the intact pelvic nerves.

There was no evidence in any of our preparations that unilateral stimulation of pelvic nerves evoked a wave of peristalsis in the wall of the bladder as has been postulated by others(5,17). In most regions the bladder response was prompt and vigorous and even in those regions of the bladder in which there was a delay in the onset of the contraction no peristaltic wave could be detected. However, it cannot be said that electrical stimulation duplicates the normal process of micturition since our observations on spontaneous micturition(18) showed a characteristic peristaltic wave passing from the proximal to the distal portions of the viscus.

Summary. Unilateral stimulation of intact pelvic nerves or of their distal cut ends, with or without section of the hypogastric nerves, usually elicited a prompt, vigorous and well sustained contraction of all portions of the detrusor muscle on the corresponding side with a weaker and often slightly delayed response on the opposite side. On 2 occasions unsustained contractions, evoked by stimulation of pelvic nerves, were observed. These data indicate that the distribution of the pelvic nerves from a given side is not limited to that side of the bladder alone but, in addition, innervates a considerable portion of the muscle on the contralateral side of the viscus.

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In vitro Turnover of Radioactive Phosphorus by Thyroid. (21489)

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The concentration of iodide by the thyroid, the synthesis of organic iodine compounds and especially the synthesis of thyroxine are presumably oxidative energy-consuming reactions (1). Since it is widely accepted that the energy derived from metabolic processes may be stored and transferred through the synthesis of compounds with high energy phosphate bonds, it seemed that a study of the phosphorus metabolism of the thyroid might give information on the energetics of synthesis of its hormone.

It was the purpose of the present study to demonstrate the ability of surviving slices of thyroid tissue to synthesize organic phosphorus compounds and to search for a possible correlation between iodine and phosphorus metabolism in the thyroid gland. Previous information(2-5) gained from studies of the iodine metabolism of surviving slices *in vitro* suggested this as a useful system for similar studies of phosphorus metabolism. Measurement was made of the effects of thiocyanate, iodide, thiouracil, thyroxine, and azide on the incorporation of inorganic P^{32} into acid soluble organic, protein, and phospholipid fractions.

Methods. Beef* thyroid or other organs were obtained fresh after sacrifice of the animals and sliced free hand with a sharp razor blade into approximately 0.3 mm. thick slices; approximately 300 mg of these slices were blotted gently on filter paper to remove excess buffer and were then incubated usually for 3 hours in 3.00 ml of Kreb's Ringer bicarbonate buffer, pH 7.4. The buffer had been saturated previously with 95% oxygen-5% CO_2 and contained 0.4 microcuries of P^{32}. The P^{32} was used directly as obtained from Oak Ridge National Laboratories,† since either different valence states of phosphorus, or pentavalent phosphorus compounds other than orthophosphate could not be demonstrated. In every experiment, unincubated tissue was analyzed as a control in the presence of the radioactive phosphate containing medium, and the activity found was used as a blank correction. After incubation, the tissue slices and medium were transferred quantitatively to a 15 ml culture tube. The beaker was washed first with two 1 ml portions of distilled water and then with 5 ml of 8% trichloracetic acid (TCA). The washings were combined with the original bath contents in the tube. This was capped, shaken for 5 minutes to extract the acid soluble material and centrifuged for 5 minutes at 2500 RPM. The supernatant was carefully decanted into a 25 ml volumetric flask. The precipitate was shaken with 5 ml of 4% TCA-.001 M Na_2HPO_4, centrifuged and the supernatant fluid decanted into the same volumetric flask. The combined contents in the 25 ml volumetric flask constituted the total acid soluble fraction. An aliquot was taken for determination of the organic acid soluble fraction. The inorganic phosphate was removed from this aliquot by the method of Rafelson *et al.*(6). After the removal of the inorganic phosphate, a 1 cc aliquot was mounted on a 1″ diameter nickel-plated steel planchet; 4 drops of 1 N NaOH were added and the sample dried under an infra-red lamp. All activity was corrected for the usual radioactivity assay variables in these and all other analyses (coincidence, background, self absorption, mass absorption, radioactive decay). The original TCA precipitate was then washed with 4 successive 5 ml portions of cold 4% TCA-.1 M NaH_2PO_4 by shaking for 5 minutes and centrifuging under refrigerated conditions to diminish hydrolytic effects of TCA. Later experiments proved the superfluity of the 0.1 M NaH_2PO_4 as a carrier in the wash liquid. After the last TCA washing of the residue, the

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* Grateful acknowledgement is made to Mr. Don Sherman of the Alpha Beta Meat Packing Co., Wintersburg Calif., who made available the beef thyroid.

† The radiophosphorus was supplied by Oak Ridge National Laboratory on authorization from the Isotopes Division, U. S. Atomic Energy Commission.

TABLE I. Comparative Synthesis of Organic Phosphorus Compounds by Thyroid, Liver and Kidney *In Vitro*.

Tissue*	Incubation period, hr	P^{32} of medium† recovered‡ as: Organic acid soluble	Protein	Lipid
		%		
Beef thyroid slices	0	.030 ± .01	.011 ± .002	.030 ± .01
" " "	3	2.66 ± .24	.78 ± .06	.31 ± .04
" " homogenate	3	—	.075 ± .026	.024 ± .008
" liver slices	3	1.09 ± .14	.177 ± .028	.212 ± .046
" kidney slices	3	1.99 ± .23	.517 ± .030	.527 ± .026
Rat liver slices	3	3.84 ± .24	.558 ± .056	1.63 ± .12
" kidney "	3	2.23 ± .09	.549 ± .024	1.23 ± .02

* In each experiment 300 mg of tissue was used.

† The medium was Krebs-bicarbonate. In each beaker 3 cc were used and contained approximately 0.4 μc P^{32} and 0.11 mg P^{31} as orthophosphate.

‡ Data represent averages and stand. dev. of experiments performed in triplicate or quadruplicate.

lipid was extracted by mixing with 10 ml of 95% ethanol. The resultant suspension was made alkaline to methyl red with a drop of concentrated NH_4OH, shaken, and allowed to stand at least one hour to dehydrate the tissue residue. After this period, centrifugation was performed, the solvent removed and transferred to a 125 ml Erlenmeyer flask. The residue was extracted 3 times further with 10 ml of 3:1 v/v of ethanol:ether (peroxide free) for 15 minute shaking periods for extraction. The extracts were combined with the dehydrating alcohol in the 125 ml flask and the mixture immediately evaporated at a low heat on a hot plate to about 2 ml. If the solution were allowed to stand a few days, an insoluble precipitate formed which contained radiophosphorus. While this could be transferred, it was somewhat difficult to remove from the flask walls. The residual liquid was quantitatively transferred to planchets lined with filter paper circles. After evaporation this constituted the phospholipid fraction. The residual from the extractions, consisting of a mixture of phosphoprotein and nucleoproteins, was dissolved by heating in an oven for 20 minutes with 0.5 ml of 2 N NaOH. This was transferred quantitatively to a planchet and counted as the protein fraction. All samples were counted to within 3% probable error (1024 counts). An aliquot of the original buffer media was also counted. All media used had the same specific activity in phosphorus. In all cases substances added for testing contributed negligibly to the total volume.

Results. Synthesis of labeled organic phosphorus compounds by thyroid slices. The formation of organic acid soluble, protein and lipid phosphorus containing compounds by thyroid slices is shown in Table I. The results are expressed as percent of labeled orthophosphate of the medium found in the organic phosphorus containing fractions after the stated period of incubation. The significant amounts of synthesis observed after 3 hours of incubation of thyroid slices are to be contrasted with the negligible values found at "zero time" for unincubated controls analyzed immediately after addition of the slices to the radioactive medium. Similarly negligible synthesis was observed after 3 hours of incubation of homogenized thyroid tissue in the presence of radioactive orthophosphate. In addition, the data demonstrate that the phosphorylative capacity of thyroid tissue, *in vitro*, is quite comparable to that of liver and kidney from both beef and rat. The *in vitro* synthesis of phospholipid by liver and kidney has been reported previously from Chaikoff's laboratory(7).

Phosphorylation as a function of time. Fig. 1 represents the increasing amounts of P^{32} incorporated into the 3 organic fractions analyzed as the periods of incubation were lengthened from 0.5 to 3 hours. Each point represents the average of triplicate incubations of 300 mg of tissue in 3 cc of bicarbonate-Ringers medium containing 0.4–1 μC of P^{32} and 0.11 mg P^{31} as orthophosphate. The appearance of the label in the organic acid soluble fraction of phosphorus compounds was most rapid in

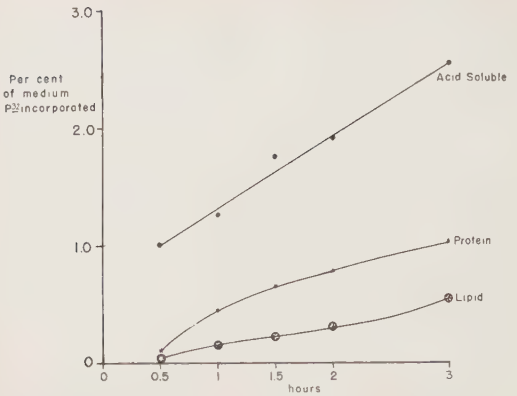

FIG. 1. Synthesis of organic phosphorus by thyroid slices as a function of time of incubation. The P^{32} was originally present in the medium as orthophosphate. Each point is avg of 3 independent experiments each consisting of 300 mg thyroid slices in 3 cc of Krebs-Ringer buffer. This volume of buffer is calculated to contain 0.11 mg P^{31}.

the first half hour; subsequent increase was essentially linear. On the other hand, the speed of appearance of label in the protein and lipid fractions is least rapid in the first half hour, but increases most significantly in the second. This increased rate continues in an essentially linear fashion in the case of the lipid fraction, but observations of the protein fraction show a progressive decline in slope.

Effect of iodide. It has been previously shown(4) that iodide at concentrations of approximately 7 μg per ml or greater had an inhibitory effect on thyroxine and diiodotyrosine synthesis under conditions similar to those used here. Table II shows no comparable effect on phosphorus metabolism with concentrations of iodide varying from 6.1 to 30.6 micrograms per ml.

TABLE II. Effect of Iodide on Phosphorus Metabolism.

Iodide added to medium,* μg/cc	Uptake† of P^{32} relative to control Acid soluble	Protein	Lipid
6.1	1.16 ± .20	1.03 ± .12	.98 ± .08
12.2	1.11 ± .06	1.09 ± .08	1.04 ± .06
18.3	1.07 ± .09	1.00 ± .14	1.00 ± .13
24.5	1.09 ± .08	1.00 ± .12	1.07 ± .14
30.6	1.09 ± .09	1.00 ± .09	1.05 ± .16

* 300 mg of thyroid slices incubated 3 hr in 3 cc bicarbonate-Ringer containing 1 μc P^{32} and 0.11 mg P^{31}.

† Results expressed as ratio of values observed in treated to those observed in controls. Deviations are stand. error of mean of 6 determinations.

Effect of thiocyanate. Franklin *et al.*(5) have shown that this substance can cause nearly complete inhibition of the uptake of iodide by thyroid tissue. It was, therefore, of interest to determine if this inhibition might not be coupled to some phosphorylative process. The results of such experiments are shown in Table III. No significant differences between treated slices and controls in concentrations varying from 0.01 to 0.05 M in thiocyanate ion were noted.

Effect of thiouracil, thyroxine and azide—Franklin *et al.*(5) demonstrated that 10^{-3}M thiouracil effectively inhibits diiodotyrosine and thyroxine synthesis *in vitro* but does not prevent the binding of iodide by thyroid tissue. In the present study (Table IV) of *in vitro* P^{32} metabolism, thiouracil in a concentration of 205 μg/cc (0.0016M) had no effect on the acid soluble and protein fractions and, if significant, only a slight stimulatory effect on the lipid fraction. Thyroxine in concentration of approximately 8 μg/cc (10^{-5}M) also had no effect. No inhibitory effect has been observed *in vitro* when similar amounts of thyroxine have been added to thyroid slices metabolizing I^{131}. However, thyroxine has been shown in this laboratory‡ to inhibit *in vivo* thyroxine synthesis in patients. Azide in 0.005 M concentration had depressant effects on P^{32} metabolism in the present studies similar to those reported in thyroid for iodine metabolism(1) and in liver slices for phosphorus metabolism(8).

TABLE III. Effect of Sodium Thiocyanate on Phosphorus Metabolism.

NaSCN molarity	Uptake* P^{32} relative to control† Acid soluble	Protein	Lipid
.01	1.01 ± .06	.92 ± .05	.94 ± .14
.03	1.09 ± .14	.96 ± .05	.94 ± .08
.05	1.04 ± .09	1.01 ± .07	.93 ± .08

* Results expressed as ratio of values observed in treated to those observed in controls. Deviations are stand. error of mean of 6 determinations.

† In each study 300 mg thyroid slices incubated for 3 hr in 3 cc of bicarbonate-Ringer medium containing 1 μc P^{32} and .11 mg P^{31}.

Discussion. That thyroid tissue can support phosphorylation, even *in vitro*, is not un-

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‡ Morton, M. E., unpublished observations.

TABLE IV. Effect of Thiouracil, Thyroxine and Azide on Phosphorus Metabolism.

Test substance conc.	Uptake* of P^{32} relative to control†		
	Acid soluble	Protein	Lipid
Thiouracil, 200 μg/cc	.98 ± .10	.99 ± .06	1.22 ± .09
Thyroxine, 8 μg/cc	1.04 ± .10	1.02 ± .13	1.10 ± .14
NaN_3, .005 M	.34 ± .16	.20 ± .17	.08 ± .10

* Results expressed as ratio of values observed in treated to those observed in controls. Deviations are stand. error of mean of 6 determinations.

† In each study 300 mg thyroid slices incubated for 3 hr in 3 cc of bicarbonate-Ringer medium containing 1 μc P^{32} and .11 mg P^{31}.

expected in view of the general distribution of phosphorus in all cells. Chaikoff(9) has summarized the evidence indicating that the major source of plasma phospholipid is the liver. Also, only small amounts of phospholipid are taken up by the other organs from the plasma (10). It is, however, of interest that the synthesis of organic phosphorus compounds by thyroid slices is as significant in amount as in liver and also is dependent on structural intactness of the tissue, being prevented by homogenization.

Although the absolute amounts of organic phosphorus compounds synthesized were not determined, the relative rates of incorporation of labeled orthophosphate into the trichloacetic acid soluble, protein and lipid fractions may be inferred from Fig. 1. At least minimal amounts synthesized may be calculated by multiplying the amount of orthophosphate in the buffer (0.11 mg) by the percentages of medium P^{32} found in the respective fractions at the various time intervals of incubation. The findings appear consistent with the hypothesis that the precursors of both protein and lipid phosphorus compounds are in the organic acid soluble fraction.

The apparent absence of depression of the *in vitro* phosphorus metabolism of the thyroid by thiocyanate and thiouracil, substances known to decrease thyroxine and diiodotyrosine synthesis is striking. In general, the absence of inhibition of phosphorus metabolism by these substances may be explained in several ways without concluding that no relationship exists between iodine and phosphorus metabolism in the thyroid gland. As an example, only a minor component or a component with low phosphorus turnover rate may be involved in iodine metabolism. The methods of analysis used in the present study may fail to detect this. A possible indication of relationship, although non-specific, may be indicated by the lack of synthesis of iodine compounds by homogenized thyroid(3) as well as the similar lack of synthesis of phosphorus compounds under the same experimental conditions. The absence of influence on phosphorus metabolism by thiouracil is, perhaps, most surprising since it might be expected to be involved in some oxidation-coupled reactions independent of any effects on iodine metabolism.

Further work on the general phosphorus metabolism of thyroid tissue is being carried out to include a study of the effects of steroid and pituitary hormones.

Summary. 1. Ability of thyroid slices to incorporate inorganic phosphate into organic phosphorus compounds is comparable to that of kidney and liver. Homogenization of the thyroid tissue before incubation prevents this synthesis. 2. A study has been made of the effect of azide, thiocyanate, iodide and thyroxine on the *in vitro* phosphorus metabolism of the thyroid. Except in the case of azide, where a general depression of phosphorus metabolism occurred, no positive correlation was found.

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Nicotinamide in Murine Tuberculosis. (21490)

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The interesting observation originally made in France(1) and remade in this country by McKenzie and coworkers(2) that nicotinamide exerts a suppressive effect on mycobacterial infections in rodents seemed deserving of further study. The relatively huge doses of the amide required for a therapeutic effect and the poor performance of the acid make the mechanism of action of nicotinamide particularly intriguing.

The original finding has been amply confirmed in this laboratory where we have been using nicotinamide during the past 5 years as a positive control in mouse chemotherapy studies. No activity could be demonstrated *in vitro* in Dubos' medium with either nicotinamide or nicotinic acid at 2 mg/cc. Efforts were made to throw some light on the mode of operation of the amide in tuberculous mice by means of the following types of experiment: 1) the dose and time of initiation of treatment were varied; 2) metabolites and other nicotinamide-like substances were administered; 3) efforts were made to obliterate the nicotinamide effect. Since these studies were begun, some experiments of a similar nature have been reported from other laboratories(3,4). In addition, synergy studies were performed with the hope that nicotinamide might have clinical application.

Procedure. The methods for preparing the inoculum and infecting the mice have been reported previously(5). An infecting dose calculated to be lethal to controls in about 3 weeks was used. Drugs were blended intimately with ground Rockland Mouse Diet. Fifty g of drug-containing diet were moistened with water and fed daily to groups of 10 mice each. Controls were given unmedicated ground diet. When the subcutaneous route of treatment was used, mice received drugs in a volume of 0.1 cc. Mice were weighed every 48 hours and observed twice a day until they died. Survival increases of treated animals in excess of 2 days appeared to have significance.

TABLE I. Results Obtained with Varying Doses of Nicotinamide.*

Dose/day, mg	Route	Increase in days' survival time over controls 50†	100†
12.5	Diet	1.2	2
15	”	1.0	2.1
25	”	10	13.3
37	”	31.4	43
5	Subcut.	1.6	6
10	”	10.2	20

* 21 days' treatment beginning day of infection.
† Results for 50 and 100% of mice, respectively.

Results. 1) Varying the dosage used produced results such as those recorded in Table I, where it will be observed that by the oral route a minimum of 25 mg per mouse per day was required to increase survival. When treatment was given by the subcutaneous route, 10 mg/day were approximately of the same order of efficacy as were 25 mg in the diet. Pretreatment, as will be seen in Table II, greatly enhanced the therapeutic effect, while delayed treatment was almost worthless. (Nicotinamide at 25 mg per day in the diet was equivalent to 0.1 mg of isonicotinic acid hydrazide, or 0.5 mg of streptomycin, or 20 mg of p-aminosalicyclic acid under the same test conditions.)

2) Miscellaneous substances that might be considered precursors, metabolites, antimetabolites, or compounds otherwise related to nicotinamide were tested (Table III). At toler-

TABLE II. Effect of Time of Initiation of Treatment with Nicotinamide.

Dose/day, mg	Route	Time treatment started with relation to infection	Total days' treatment	Days' increased survival
10	Subcut.	36 hr b*	1.5	7.6
25	Diet	48 ” b	2	4
25	”	2 wk a	7	1.4
37	”	24 hr b	21	52.8
37	”	48 ” b	9	26.8
37	”	48 ” b	22	78.8
37	”	1 wk a	14	4.2

* b = before; a = after.

TABLE III. Effect of Compounds Related to Nicotinamide.

Compound	Dose, mg	Route	Days' increased survival
Nicotinamide	25	Diet	9.4
Nicotinic acid	37	"	2.
	25	"	1.5
	10	Subcut.	−1.
N-methyl nicotinamide	37	Diet	.6
Trigonelline acid sulfate	50	"	−1.2
Ethyl-2-hydroxy-4,6-dimethyl nicotinate	25	"	−1.4
Coumalic acid	25	"	2.8
N,N-Diethyl nicotinamide	2	Subcut.	− .4
1 Methyl-3-carboxyl amide-6-pyridone	10	"	1.5
Nicotinuric acid	10	"	2.3
6-Hydroxynicotinic acid	10	"	0
6-Hydroxynicotinamide	25	Diet	2.4
Ethyl nicotinate(2)	5	"	−1.5
Methyl "	5	"	−1.
Tryptophane(2)	37	"	0
Pyridoxine	5	"	0
2-Aminopyridine	12.5	"	0
Pyridine-3-sulfonic acid	10	Subcut.	.4
3-Acetyl pyridine(4)	2.5	"	0
Quinolinic acid	10	"	−2.4 (toxic)

ated doses, no substance among those that might have been expected to do so had any striking effect. The prolongation of life of mice given nicotinic acid in the diet was negligible, a result that was not improved by subcutaneous treatment. Treatment with quinolinic acid resulted in a slight increase in susceptibility, though this may have been merely a reflection of the inherent toxicity of this compound. Adenine, guanine, glutamic acid, and pyridoxine also were tried and found to be ineffective. It seemed reasonable to suppose that nicotinamide functioned protectively because of its relation to coenzyme I. To test this hypothesis, pure coenzyme I was given to mice subcutaneously alone and in combination with nicotinamide in the diet. Coenzyme alone did not prolong life, but the combined treatment increased survival by ten or more days over that of mice treated with nicotinamide alone, provided that treatment was started the day of infection.

3) Our knowledge of the metabolism of nicotinamide in the mouse is less complete than for other animals. However, since methylation is recognized as a major step in nicotinamide metabolism in man and in the rat, the possibility existed that large doses of this agent were effective in mouse tuberculosis because they produced a methyl group deficiency in the host that might be unfavorable to the multiplication of the tubercle bacillus. Some compounds containing methyl groups therefore were fed in large amounts with and without nicotinamide to see if the favorable effect could be counteracted. From Table IV it can be noted that the substances alone were non-toxic and that they did not detract from the nicotinamide effect. (Rogers *et al.* have made the same observation(4).) Choline, actually, seemed to enhance the nicotinamide effect.

During the course of these experiments, it occurred to us that the cornmeal diet used by Dubos and Pierce(6) for enhancing the susceptibility of mice to tuberculosis might do so at least in part by producing a tryptophane (and therefore a nicotinamide) deficiency. We were able to counteract the deleterious effect of the cornmeal diet by the concurrent administration of nicotinamide. However, this does not constitute clearcut proof for the mode of action of cornmeal because of the favorable effect of nicotinamide alone. We were not successful in an attempt to increase the susceptibility of weanling rats by feeding the cornmeal diet, a result that may be related to the high total nicotinic acid content of the tissues of this animal(7).

It was obvious that if doses of nicotinamide equivalent to those needed to increase survival in mice were required to treat human beings, this agent could have no practical application.

TABLE IV. Effect of Administration of Substances Containing Methyl Groups.

Treatment*	Dose/day, mg	Days increase in 50% survival time
Ch	25	2
Ch + N	25 + 37	40
N	37	31.4
dl M	75	0
dl M + N	75 + 25	7.6
N	25	8.6
E	25	.8
E + N	25 + 37	40
N	37	37.2

* Ch = Choline; N = Nicotinamide; M = Methionine; E = Ethanoleamine.

(In fact, treatment of man has been reported (8,9).) Attempts to demonstrate a synergistic effect with known tuberculostatic drugs therefore were made. Mice were given nicotinamide combined with streptomycin, or tibione, or isonicotinic acid hydrazide, or p-aminosalicyclic acid. No synergy occurred with any of these agents. It is interesting to note that there was no antagonism between nicotinamide or nicotinic acid and isonicotinic acid hydrazide.

Discussion. McKenzie and her associates (2) felt that they might be dealing with a vitamin effect, but because of the large doses required it appeared to us more likely that a metabolic product of nicotinamide was the active agent, or that a methyl group deficiency produced in the mouse accounted for the favorable result. Experiments have not borne out these theories.

That nicotinamide exerted its effect because of its relation to coenzyme I was an attractive possibility. It has been shown by Williams that the levels of total nicotinic acid (nicotinic acid, nicotinamide, coenzyme) for man and the mouse in all tissues assayed were lower than for the rat(7), an animal highly resistant to experimental infection with the tubercle bacillus. Further, the total nicotinic acid values for lung for all species examined were among the lowest figures obtained in the tissue assays. Axelrod and Elvehjem(10) found the coenzyme I content of the tissues of the guinea pig to be lower than that of the rat or dog. We were not able to demonstrate a therapeutic effect with coenzyme I given alone, but found it very effective when combined with nicotinamide. (Mann and Quastel have pointed out that the breakdown of coenzyme I can be inhibited by nicotinamide(11).)

The striking prolongation of life observed when mice were treated even for only a short time before infection might point perhaps to a mechanism in which nicotinamide exerts its effect on the *host*, creating an environment unsuitable for the rapid multiplication of the tubercle bacillus. It is interesting to speculate on whether the nicotinamide level may contribute to the resistance of certain species and whether it might account in part for the variation in susceptibility among human beings. It may be fortuitous and obviously does not account for all the factors involved, but there is a relation between susceptible groups in human populations (nations during wartime—negroes of southern U.S.) and nutrition with high-nicotinamide foods.

Although the method by which nicotinamide produces its favorable result has not been satisfactorily elucidated, its relation to coenzyme breakdown makes this mode of action a likely possibility. The ability of nicotinamide to prolong the life of mice, particularly when treatment is begun before infection, may indicate that an ample intake of foods rich in this vitamin plays a role in the resistance of human beings to tuberculosis.

Summary. Experiments were performed to assess the therapeutic effect of varying the dose and the time of initiation of treatment with nicotinamide in tuberculous mice. No activity was demonstrated for any substance tested that is related to this agent. It was found that treatment instituted prior to infection exerted a striking effect. Therapy with coenzyme I in addition to nicotinamide greatly increased survival. No synergy occurred with known tuberculostatic drugs.

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Phase Contrast Cinematography of Cellular Lesion Produced by Poliomyelitis Virus *in vitro*. (21491)

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A previous study of the characteristics of the lesion produced by poliomyelitis virus *in vitro* in fixed and stained preparations of human adult fibroblasts indicated that an eosinophilic area, which becomes soon more precisely outlined, appeared in the perinuclear cytoplasm between 15 and 20 hours after infection(1). As this eosinophilic mass increased in size in the center of the cell, the nucleus and the cytoplasm, which became increasingly basophilic in content were pushed to the periphery. At the next stage, the cell progressively emptied itself of its cytoplasm. We have since observed a similar lesion in fixed and stained preparations of fibroblasts derived from human embryonic muscle and of human and monkey kidney epithelial cells, infected with poliomyelitis virus. The increasing basophilia of the virus-infected cell was found to be associated with a strongly positive and generalized Gomori alkaline phosphatase reaction. The purpose of the present communication is to describe the evolution of the cellular lesion as it was observed by phase contrast cinematography without fixation or staining.

Materials and methods. Strains of fibroblasts derived from human tonsils were maintained by serial passage in roller tubes for 2 to 3 months before they were used. These cells are 3 to 4 times as large as the average skin-muscle fibroblasts, regular in shape, and remarkably resistant to nonspecific degeneration. Their susceptibility to poliomyelitis virus was reported in a previous communication(2). For examination with the phase contrast microscope explants were prepared by the hanging drop technic. The medium consisted of chicken plasma, human serum free of antibodies for the viruses that were used, bovine amniotic fluid, Hanks' solution and traces of chick embryo extract. The MEF_1 strain of Type 2 virus and the Mahoney strain of Type 1 poliomyelitis virus were used and it may be noted that the lesions were the same with both. Before transplantation to the hanging drop the cells were mixed with virus suspensions having a titer of 10^{-5} and left at room temperature for 30 minutes. Observations were carried out at 36-37°C and at 30-31°C. The evolution of the lesion was slower at 30-31°C but otherwise the same at both temperatures. The lower temperature was used more often because the control cells remained in better condition at 30-31°C. The observations were recorded with a Zeiss-Winkel phase contrast microscope at magnifications of 240X, 540X and 900X, using a 100V tungsten, ribbon lamp for the light source. The moving picture system of the camera was the one designed by J. Frédéric (3); pictures were taken every 2 to 5 seconds.

Results. The cellular lesion produced by poliomyelitis virus is portrayed in Fig. 1-15 representing different frames from the complete cinematographic study by Robineaux, Barski and Endo. Fig. 1 shows an essentially normal fibroblast. The first signs of disturbance of the normal cellular architecture appear at 30-31°C in about 20 hours after infection in the form of a thickening of the perinuclear area. During the next 3 to 5 hours, a round or oval mass, which indents and overlaps the nucleus, gradually appears in the center of the cytoplasm (this mass can be seen to the right of the nucleus in Fig. 2 and 3). This central mass appears granular and more compact than the cytoplasm. Neither mitochondria nor lipoid drops are seen inside this mass and vacuoles are only seldom present. While the constituents of the surrounding cytoplasmic area exhibit active movement, the central mass remains practically motionless and suggests greater density and stiffness. As this cytoplasmic mass increases in size the nucleus is pushed to the periphery of the cell and begins to show signs of damage. The nuclear outline becomes irregular and less distinct and the

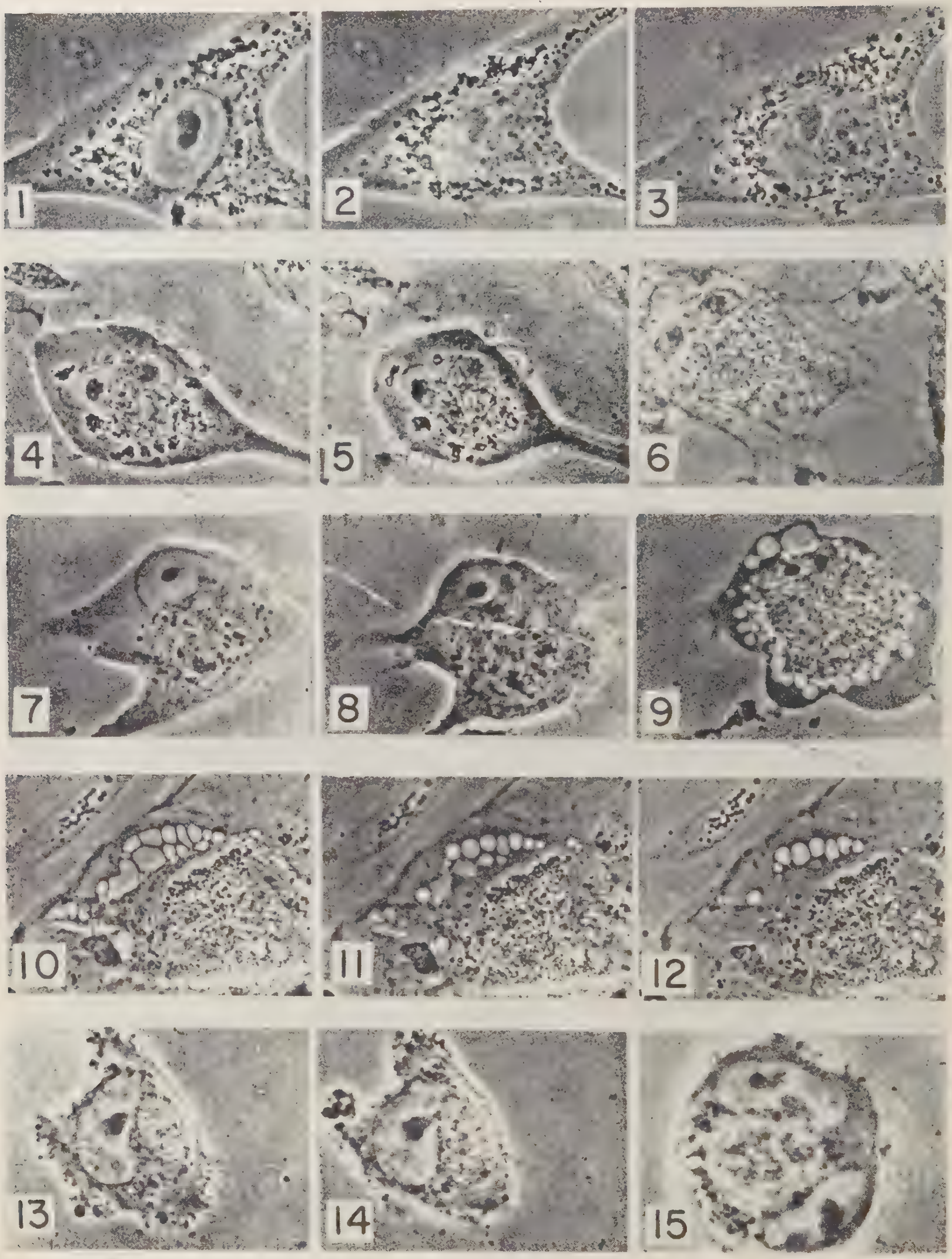

FIG. 1-14, ×820. FIG. 15, ×1050.

nucleoli appear distorted. This lesion does not suppress the vital activity of the cell, which retains its mobility and exhibits filamentous mitochondria at the periphery of the cytoplasm. At the next stage cytoplasmic protrusions appear with a quick rhythm all around the cell outline, similar to the "bubbling" phenomenon observed during the telophase in mitosis of a normal cell. After this "bubbling" stage the cell comes to a standstill. The outer layer of the cytoplasm displays a characteristic vacuolization which is followed by a gradual disintegration of the cellular outline in about 40 to 50 hours after infection. Such a pattern of lesions and changes was never seen in the control, uninfected cells even when they developed nonspecific degeneration ending in death.

Fig. 4, 5 and 6 show one cell at 30, 43 and 53 hours after infection. The appearance of another cell at 24, 28 and 32 hours is shown in Fig. 7, 8 and 9. During the contractions of this cell portions of the cytoplasm became detached and lost in the medium (see center bottom of Fig. 8 and 9); note also the extensive vacuolization of the cytoplasm surrounding the central mass in Fig. 9. Further details of this vacuolization are shown in Fig. 10 and 11 which were photographed at an interval of 4 minutes and 15 seconds and in Fig. 12 which was photographed 2 minutes and 50 seconds after Fig. 11. It can be seen that the vacuoles change and disappear during the course of a few minutes. Fig. 13 and 14 show another cell at 40 hours after infection: the well-circumscribed central mass indenting the nucleus and the change in marginal cytoplasm resulting from active "bubbling" movement during the 25-minute interval between photographs, are seen clearly. Fig. 15 shows a binucleated cell with a well-formed central mass between the 2 nuclei.

Discussion. The exact nature of the dense and motionless mass which appears in the center of cells infected *in vitro* by poliomyelitis virus remains obscure. There is no proof that it represents the site of virus synthesis or maturation, although certain data(4,5) suggest that poliomyelitis virus is produced predominantly, if not exclusively, in the cytoplasm. It has been suggested that *in vitro* poliomyelitis virus begins to be released from infected cells at an early stage before the final destruction of the cell, and the quantitative data of Dulbecco and Vogt(6) indicate that the mechanism is different from the release of bacteriophage by bursting bacteria. During the period corresponding to the earliest release of poliomyelitis virus, the cinematographic pictures show that the cell still exhibits active movements often accompanied by a violent, bubbling phenomenon in the outer layer of the cytoplasm. Moreover, this is followed by an appearance and disappearance of vacuoles in the same area of the cytoplasm. It is possible that these phenomena may be associated with the release of poliomyelitis virus by the still living cell.

Summary. Cultures of adult, human fibroblasts of relatively large size and high resistance to nonspecific degeneration were infected with Types 1 or 2 poliomyelitis virus and submitted to continuous observation by means of phase contrast microcinematography. A central dense and motionless mass regularly appeared in the cytoplasm. As this mass increased in size at the expense of the cytoplasm the nucleus was pushed to the periphery of the cell, but the remaining cytoplasm continued to exhibit signs of vital activity. The "bubbling" and vacuolization of the marginal cytoplasm observed in the infected cells are considered as representing a possible mechanism for the release of virus some hours before the ultimate destruction and death of the cells.

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Determination of Sodium Content of Human Sweat by Radioactive Sodium, (Na^{24}).* (21492)

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(Introduced by Saul J. Farber.)

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Dole, Stall, and Schwartz(1), have presented a method for the quantitative determination of the sodium concentration of human sweat derived from small local areas. A local flow of sweat is induced by the subdermal injection of mecholyl chloride, and the sweat so produced is collected on small filter paper discs. These discs are held to the skin by means of an air tight ring-like container which prevents evaporative loss of sweat as it is being collected. The amount of sweat produced is determined by the increment in weight of the collecting filter paper discs, and the sodium content by elution from the paper and determination by the flame photometer.

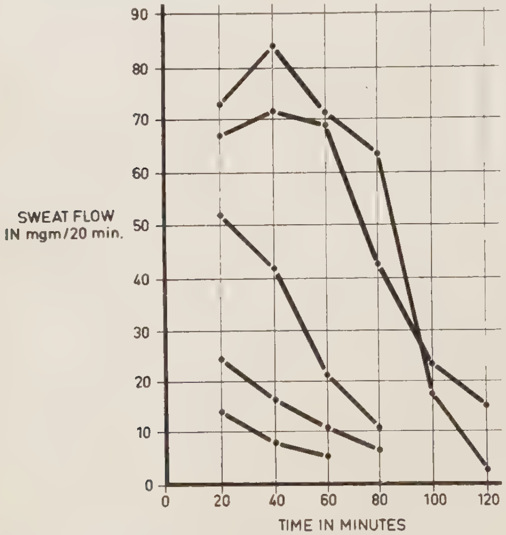

FIG. 1. Time-flow relationships. Dose was 2 mg of mecholyl chloride. Abscissa: Time in min. from time of mecholyl inj. Ordinate: Rate of sweat flow.

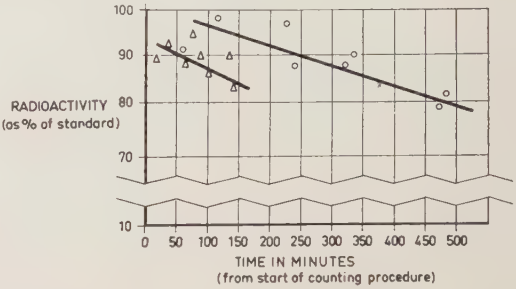

FIG. 2. Recovery of Na^{24} from wetted filter paper discs in contact with non-sweating skin. Triangles represent radioactivity of discs, wetted with known quantities of radiosodium, after 20 min. exposure to non-sweating skin of patients previously inj. with Na^{24}. Circles represent identically prepared but unused standards. Representation of semi-logarithmic graph paper.

The method here presented is a variation of the above technic in that it utilizes the detection of radioactive sodium as a means of measuring the sodium content of sweat.

Method. On the night preceding the determination, the patients are given an intravenous injection of 100 μc of radioactive sodium (Na^{24}). On the following morning the chosen skin areas are washed with alcohol to remove surface accumulations of sweat, and are allowed to dry. Thus far the inner surfaces of the arms and thighs have been used as collection sites. The aluminum devices designed by Schwartz, Thaysen, and Dole(2) are then attached at their ring-like edges to the skin with Duco cement. The copper planchettes which fit inside the rings, and upon which the sweat is collected, are prepared in advance by attaching with rubber cement to one surface of each planchette a Whatman No. 1 filter paper disc of the same size. Each planchette is then placed in a small crucible, covered with parafilm, and weighed. The prepared planchettes are hereafter always handled with forceps. Mecholyl chloride[§], 2 mg in 0.5 cc

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[‡] Drs. Genkins and Braunwald: Mount Sinai Hospital, N. Y. City.

[§] Mecholyl chloride was supplied through the courtesy of Merck Inc.

of a 1% procaine solution, is injected subdermally into the center of each aluminum ring. A planchette is removed from its crucible, and inserted into a ring so that the filter paper is in contact with the skin. The top plug is placed on the planchette and then rapidly screwed down so that the ring is sealed. The collecting devices are opened after 20 minutes, the planchettes are removed, new planchettes are inserted, the ring is sealed, and a second 20-minute collection period is begun. Immediately after removal from the collecting device, each planchette is replaced into the same crucible in which it was previously weighed, the parafilm cover is tightly reapplied, and the entire unit reweighed. Working at moderate speed, but without undue haste, a fairly constant loss of 2 mg of sweat from each planchette has been shown to occur during the transfer from the collecting device to the weighing crucible. The increment of weight of the unit, (planchette, crucible, and parafilm cover), plus 2 mg to allow for the evaporative loss during transfer, gives weight of sweat collected. After the weighing, the planchette is removed and allowed to dry in air. The sweat produced in response to a single injection of mecholyl, is collected for from 3 to 6 collection periods of twenty minutes each. The *radioactivity* of the filter paper (on the planchette) is due to the radioactive sodium of the absorbed sweat and is determined by a Geiger-Muller counter. For each sample, the mean value of 2 successive determinations is used. A sample of 0.2 cc of the patient's serum, drawn just after the sweat collections, is plated on a similar filter paper disc attached to a copper planchette. Another 0.2 cc serum sample obtained several hours after the collections is similarly treated. The radioactivity of each of these serum samples is determined 4 to 5 times. If all of the points for the two serum samples fall on the same "decay" curve, then equilibrium of distribution of the injected radioactive sodium in the patient's "sodium space" may be assumed to have occurred. The serum sodium of the sample drawn just after the sweat collections is then determined by means of an internally standardized flame photometer.

Sodium concentration of sweat collected is determined according to the following formula: Sweat Sodium Conc. (in mEq/L) = $\frac{\text{Scpm x 0.2 x Na}}{\text{Bcpm x Wt}}$ where Scpm = Counts/minute, minus background, from sample of sweat; Na = Serum sodium concentration in mEq/L, determined by flame photometer. Bcpm = Counts/minute, minus background, from 0.2 cc of serum, interpolated to correspond in time with sample of sweat being analyzed. Wt. = Increment of weight, in grams, of planchette after sweat collection, corrected by addition of 2 mg to account for evaporative loss in handling before weighing.

Results. The sodium concentration of sweat from local areas has been determined by this method in 11 patients, and in 9 of them at 2 or more sites simultaneously, (Table I). The amount of sweat flow induced by subdermal injections of mecholyl chloride varied widely in different patients and at different sites. During the first 20 minutes after the injection, the sweat produced varied from 8.5 to 82.0 mg. The flow usually reached its peak in the first or in the second 20 minute period, and then fell gradually during ensuing periods (Fig. 1). The lowest sweat sodium concentrations ranged from 8 to 12 mEq/L, (a patient with hyperthyroidism), and the highest range was from 90 to 120 mEq/L, (a patient with resolving lung abscess).

Na^{24} Recovery Experiments: The exchange of both sodium ion and water between the skin and the filter paper disc under conditions comparable to the experimental conditions was determined by the following experiments: a) Dry discs containing a known amount of radioactive sodium, attached to planchettes (handled as described above), were put in contact with skin of a patient not previously injected with Na^{24}. Local sweat flow was induced by the subdermal injection of mecholyl chloride. After the usual 20 minutes, the planchettes were removed and their remaining radioactivity counted and compared to identical but unused standards. In 9 such determinations, the loss of radioactive sodium from a disc-planchette unit to the sweating skin averaged less than 5%. The values of highest loss obtained were not correlated with higher rates of flow

TABLE I. Local Sweating: Flow Rates and Sodium Concentration in 11 Patients. Flow is measured in mg of sweat/20 min. collection period, and concentration of sodium is in mEq/L (to the nearest unit). Dashes indicate insufficient radioactivity. Samples in the lower ranges of sodium concentration (5-15 mEq/L) required "flows" of at least 15-20 mg for accurate determination. Dose of mecholyl chloride was 2 mg at each site.

		Collection periods							
		1		2		3		4	
Diagnosis	Collection site*	Flow	Conc.	Flow	Conc.	Flow	Conc.	Flow	Conc.
Pulmonary fibrosis & emphysema; Cor Pulmonale	RA	57.9	31	35.4	24				
	LA	64.5	37	28.0	33				
Bronchogenic carcinoma; hypertensive cardiovascular disease	RA	64.5	61	75.8	37				
	LA	61.3	55	61.8	52				
Pulmonary fibrosis & emphysema; (post cortisone therapy)	RA	72.3	19	82.3	13	68.4	16	62.9	13
Hyperthyroidism	RA	66.4	13	71.6	10	66.8	9	42.0	—
Infiltrative pulmonary lesion	RA	24.1	79	17.7	70	11.8	59		
	LA	22.1	79	22.2	70	13.1	62	9.2	—
Gouty arthritis in remission	LA	34.2	63	30.5	61				
	RL	14.8	61	13.3	60				
	LL	8.5	51	5.2	—				
Bronchogenic carcinoma	RA	12.3	15	16.3	28	14.8	37	46.5	52
	LA	26.7	42	24.2	34	17.7	32	50.5	37
Peptic ulcer	RA	41.5	67	32.4	75	21.4	66	10.9	52
	LA	48.5	88	41.5	84	20.8	61	6.6	—
Gouty arthritis in remission	RA	50.9	29	41.1	24	20.3	20	11.4	—
Resolving lung abscess	RA	40.4	113	36.8	122	16.3	93	3.5	—
	LA	36.0	122	22.5	85	10.5	100	5.5	87
Chronic glomerulonephritis	RA	32.0	35	25.0	24	41.6	18	40.2	22
	LA	42.1	42	23.4	44	18.2	52	9.0	—

* RA = right arm; LA = left arm; RL = right leg; LL = left leg.

of sweat. b) Determination of the gain or loss of radioactive sodium to the non sweating skin: Planchette-disc units wetted with a solution (100 mg) of known radioactive sodium concentration, were placed on the dry skin of patients whose body fluids had come into equilibrium following an intravenous injection of 100 μc of Na^{24}. The planchettes were handled in the usual manner, and left in contact with the non-sweating skin for the usual 20 minutes. They were weighed before and after the skin contact, and their radioactivity was measured after skin contact. An identically prepared but unused standard served for "decay" determination. Eight determinations showed the loss of radioactive sodium from a wet planchette to the non sweating skin to be approximately 10%. (Fig. 2).

Comparative accuracy of method. Urine samples were obtained the morning after day of sweat collections, and 0.05 to 0.1 cc, plated on the same filter paper-planchette units as used for sweat collections, and handled like a sweat sample. The value of urine concentration of sodium as determined by the "radioactive" method was compared to that obtained by flame photometry on another aliquot of same urine sample. In 9 such paired determinations the urinary sodium concentrations agreed within 5%.

Dose-flow relationship. Four separate sites on a single patient were selected, one on each arm, and one on each thigh. Four different doses of mecholyl chloride (0.5, 1, 2, and 3 mg) were used, one dose at each site. The total dose for the determination was 6.5 mg. On 4 successive days the same dosage schedule was used and individual doses rotated through the 4 sites, so that by the end of 4 days each site had been stimulated by each of 4 doses, with the same total systemic stimulation each day. The data of the dose-flow curves (Fig. 3) indicate that while the rate of sweating on the arm increased

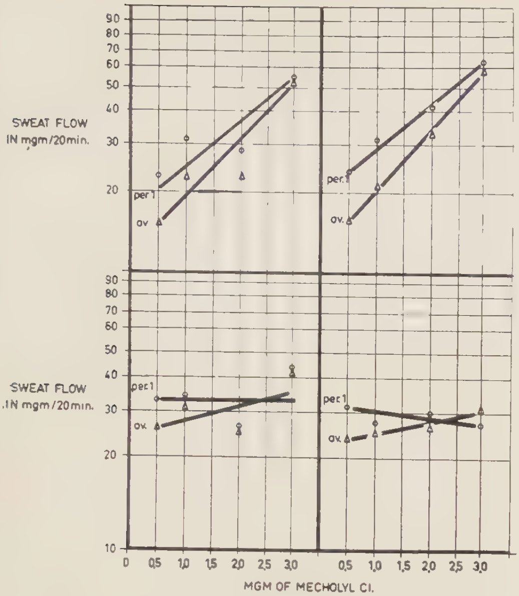

FIG. 3. Local sweat response to varying dosage of mecholyl chloride inj. subdermally. Each section shows values for an independent site on body. Upper 2 sections represent the arms, lower 2 the legs. Abscissa: Dose of mecholyl. Ordinate: M rate of sweat flow. Circles represent the first period response at each site. Triangles describe avg response for 3 periods at each site and for each dose. Representation of semi-logarithmic graph paper.

in response to increasing doses of mecholyl, the sweating rate in the thigh remained relatively unchanged.

Discussion. The chief advantage of the method using radioactive sodium is its accuracy. The same method previously described (1), but using flame photometric analysis for determination of sodium concentration of samples of sweat, gave in our hands errors of 50-60% when sweat flow rates were low. As modified by the use of Na^{24} this method has an error of approximately 5%, and furthermore eliminates the necessity for siliconed glassware, blank determinations, and the trying and not always possible preparation of sodium free equipment. For the purpose of determining sweat sodium, 2 mg of mecholyl chloride has been found an adequate sweat stimulating dose, and the arms the preferable collection site.

When 0.05 to 0.15 cc of sweat obtained during one collection period are diluted to the volume necessary for flame photometry, the accuracy of such a determination is markedly reduced. In urine, there is sufficient sodium in amount and concentration for the use of both "radioactive" and photometric methods without the difficulty of such a dilution factor. In 9 successive determinations of urinary sodium by the two methods, the results differed by an average of 2% with no single pair differing by more than 5%.

The loss of radiosodium from the wetted filter paper to the non-sweating skin was approximately 10% and probably was due to the film of fluid retained on the previously dry skin. However, this condition is not present in the usual experimental procedure where a dry piece of filter paper is placed on sweating skin. Less than 5% of radioactive sodium placed on dry filter paper discs was lost to the sweating skin. The dry filter paper thus apparently effectively absorbs the sweat and its contained electrolyte.

It has been suggested that concentration of sodium in sweat is directly proportional to rate of flow of sweat(1,3). Although the present observations are insufficient for a valid appraisal of this point, the sodium concentrations of the first period (when sweat flow was high) tended to be higher than in successive periods. (It is true that this may be a value falsely raised by partially inspissated sweat present in many gland ducts.) Our successive collection periods showed no closely parallel fall of sodium concentration as rate of sweating sharply decreased. Also(4) during heat adaptation studies when enormous sweating rates represented a very significant factor in body fluid and electrolyte economy, sodium concentration of this sweat was extremely low.

Methods for local induction of sweating for electrolyte determinations present several advantages. By avoiding the use of generalized body heating, these methods assure the cooperation of even severely ill patients who are poorly able to stand general heat. Moreover, through heat adaptation function of the adrenal-pituitary interrelationship, generalized body heating may rapidly alter the electrolyte concentration of sweat(4,5). It also appears that chemically induced local sweat does not differ significantly from the more physiological thermal sweat in composition or other features(6).

Summary. A method for determination of sodium concentration of sweat from local areas is described. It consists of administering radioactive sodium to subjects, and then inducing a local flow by subdermal injections of mecholyl chloride. Sweat so produced is collected by the method of Dole, Stall and Schwartz, employing filter paper discs sealed in a collecting device. Sodium concentration is derived by determining the radioactivity of the filter paper, and its increment in weight after sweat collection.

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Difference in Susceptibility to Ak Leukemic Agent Between Two Substrains of Mice of C3H Line.* (21493)

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In previous experiments, centrifuged or filtered leukemic extracts, prepared from livers, spleens, and lymphoid tumors of leukemic mice of strain AK-n, or C58, inoculated into newborn mice of the C3H inbred line, resulted in development of either leukemia, or salivary (parotid) gland tumors(1-6). When these experiments were repeated at the National Cancer Institute under apparently similar conditions, it was noticed that following inoculation of newborn C3H mice with either centrifuged or filtered leukemic extracts, parotid gland tumors could be induced with no difficulty. However, very few animals among inoculated C3H mice developed leukemia(7). It appeared of interest, therefore, to determine whether a difference in susceptibility of substrains of C3H mice, might have been responsible for this difference.

Two substrains of mice of the C3H inbred line have been used in our laboratory. One strain consisted of descendants of C3H mice originally obtained from J. J. Bittner, University of Minnesota, in 1944(8), and bred brother-to-sister mating. These mice (carrying the milk-transmitted mammary carcinoma agent) are designated by the symbol C3H. A separate colony(1) has been simultaneously raised (by brother-to-sister mating) from a foster nursed litter of the same substrain, and designated by the symbol F. Another substrain of the C3H mice, (free from the milk agent by foster nursing), was obtained in 1945 from H. B. Andervont, National Cancer Institute. These C3H mice have been also bred brother-to-sister mating, and the colony raised from this particular substrain was designated by the symbol An(8). Since spontaneous mammary carcinomas(9), or induced leukemias(6), developing in one substrain, can be transplanted without difficulty into adults of the same, but not into the other substrain, it is apparent that these 2 substrains are genetically different.

In recent experiments at the National Cancer Institute, only C3H mice of the Institute's substrain (apparently genetically identical with our An mice) were used for inoculations of cell-free leukemic extracts(7). Since we did not suspect any difference in susceptibility to cell-free leukemic agent between these 2 substrains, both substrains were used for inoculations. In our recent publications(1-5), all C3H mice, of both substrains, were grouped together, without individual substrain distinction. Proper identification of

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TABLE I. Results of Inoculation of Filtered Ak Leukemic Extracts into Newborn (Less than 16 Hours Old*) C3H Mice of Two Distinct Substrains.

			Inoculation of FRESH leukemic extracts					Simultaneous inoculations of HEATED (65° to 68°C ½ hr) Leukemic extracts into litter-mate control mice				
Substrain C3H	Symbol used	Filter	No. of mice inoc.	No. of mice devel. leuk.	Avg age leuk. devel., mo	No. of mice devel. parotid tumors†	Avg age parotid tumors devel., mo	No. of mice inoc.	No. of mice devel. leuk.	Avg age leuk. devel., mo	No. of mice devel. parotid tumors	Avg age parotid tumors devel., mo
Nat. Cancer Institute	An	B‖	34	0		4	11	28	0		0	
		S	128	7	14	5	12	17	0		0	
	Total		162	7 (4%)		9 (6%)		45	0		0	
Bittner	C3H‡	B	40	13	8	7	5	29	0		0	
		S	98	19	11	17	6	44	0		1	8
	F	B	59	14	11	1	3	53	0		3	4
		S	123	44	10	8	5	40	2	17	1	4
	Total		320	90 (28%)		33 (10%)		166	2		5§	

* Most mice were <12 hr old, many <6 hr, none >16 hr at time of inoculation.

† Among mice which developed parotid gland tumors, several also developed subcutaneous fibrosarcomas, usually near the groin, axilla, or within the abdominal wall. In one mouse, a medullary adrenal tumor was also observed. Two mice developed, in addition to parotid gland tumors, carcinomas of the submaxillary gland, arising in small multiple foci. In one instance bilateral parotid gland carcinoma developed simultaneously with leukemia.

‡ Among C3H females of the Bittner substrain, carrying the mammary tumor agent, many developed spontaneous mammary carcinomas. These tumors are not included in the Table.

§ Since spontaneous parotid gland tumors are extremely rare in untreated C3H mice (we can recall only 1 among several thousand C3H mice, observed during the past 10 years), it is of interest that 5 mice among those inoculated with heated leukemic extracts developed parotid tumors. This could be explained either by contact infection, or, more probably, by assuming that leukemic extracts required a higher temperature for complete inactivation.

‖ B = Berkefeld; S = Selas.

substrain was, however, recorded in our protocols.

In view of Law's experiments(7), we reexamined our previous and current protocols to determine whether any difference existed in susceptibility between these 2 substrains to leukemogenic action of the Ak agent. Accordingly, protocols (in part published) of filtration experiments were reviewed; the results were tabulated in Table I. Of 320 mice of the Bittner substrain, 28% developed leukemia. Of 162 mice of the National Cancer Institute substrain only 4% developed leukemia. It became apparent that C3H mice of the Bittner substrain are substantially more susceptible than mice of the National Cancer Institute substrain to leukemogenic action of filtered Ak leukemic extracts. This unexpected difference between these two C3H substrains may in part explain the apparent difficulty in inducing leukemia in experiments in which C3H mice of the National Cancer Institute's substrain were used. It is also possible that other investigators using C3H/Jax mice obtained recently from the Jackson Memorial Laboratory, Bar Harbor, may also be dealing with the same National Cancer Institute's substrain(10). Such investigators may have the same difficulty in inducing leukemia, unless they use Bittner's substrain for inoculation of the Ak agent.

It remains to be determined whether the leukemic agent could be recovered as readily from the Ak (substrain AKR-RIL) mice used at the National Cancer Institute, as from those used in our laboratory(8) (substrain Ak-n). Another factor which should be considered, in determining whether inoculated C3H mice will develop leukemia or parotid gland tumors, is the proper concentration of leukemic extract inoculated. Centrifuged (7,000 x g) Ak leukemic extracts of 20% concentration, uniformly induced leukemia when inoculated into newborn C3H mice of the Bittner substrain(3). A further 10-fold, in some experiments 100-fold dilution of such extract, induced frequently salivary gland tumors, instead of leukemia(5,6). Furthermore, to obtain positive results, only newborn mice less than 12 hours, and preferably less than 6 hours, should be used for inoculations. When older mice are injected, the results are frequently either considerably delayed, or negative.

Summary. 1. Of 320 C3H mice (substrain Bittner), inoculated with filtered Ak leukemic extracts, 28% developed leukemia. Of 162 C3H mice (substrain National Cancer Institute) inoculated simultaneously with the same extracts, only 4% developed leukemia. 2. The incidence of parotid gland tumors, induced with the leukemic extracts in these 2 groups of C3H mice, was 10% vs. 6% respectively. 3. Newborn C3H mice of the Bittner substrain are, therefore, substantially more susceptible to leukemogenic action of the Ak leukemic agent, than those of the National Cancer Institute substrain, of the same line.

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Stability of an Azoprotein Hapten in the Organism.* (21494)

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During the last few years azoproteins prepared from S^{35}-sulfanilic acid or C^{14}-anthranilic acid have been used as trace labelled antigens. The radioactivity of these substances, after injection into rabbits(1,2) or mice(3) is rapidly cleared from the blood and is incorporated into the cytoplasmic granules of liver and spleen where it persists over many months. The determinant groups of these heavily substituted azoproteins, as shown by the classical work of Landsteiner(4), are their aromatic residues. Thus, injection of serum γ-globulin coupled with diazotized sulfanilic acid (sulfanilazo-BGG) leads to the formation of antibodies which combine with the *p*-azophenylsulfonate group.

It is important to know whether the radioactivity found in the tissues is that of the unchanged hapten, or whether the hapten is degraded and its S^{35} incorporated into the amino acids of the tissue proteins. In order to decide this question, the liver protein of rabbits injected with S^{35}-sulfanilazo-BGG was examined for radioactivity in the cystine and methionine fractions, and an attempt was made to isolate the material bearing the radioactive label.

Methods. S^{35}-sulfanilic acid, prepared according to Ingraham(5), was diazotized and coupled to beef serum γ-globulin (Armour) by the usual procedure(1). The azoprotein was injected intravenously into rabbits in a single dose of 30 mg/kg body weight. After killing the animal, the liver was passed through a Latapie mill. A 10% (w/v) suspension was made of the liver in a 0.88 *M* sucrose solution. The protein was then precipitated and a dry powder was prepared as described earlier(6). Approximately 30 mg of the dry liver protein or its fractions were counted. When the material to be counted was less than 30 mg, it was mixed with adequate amounts of normal beef serum and dried; 30 mg of this powder were then counted. All counts reported were obtained with an RCL Q-gas flow-counter, and were corrected for self absorption and decay.

Results. Quantitative analyses‡ of the liver protein of animal killed 2-4 weeks after injection of the S^{35}-azoprotein revealed that the alkali labile sulfur(7) of the liver protein and the methionine fraction, prepared according to Ehrlich-Gomolka(8), contained only 2 to 5% of the total radioactivity.

Isolation of cystine and methionine. Rabbit No. 277, injected intravenously with 60 mg S^{35}-sulfanilazo-BGG containing 7.7×10^6 counts per minute, was killed 4 weeks after injection. The total weight of dry liver protein was 13.25 g of which 30.3 mg gave 151.2 counts per minute. This corresponds to approximately 0.5 mg of the injected azoprotein in the total liver. From 6.63 g of the protein, cystine and methionine were prepared according to Williams and Dawson(9) except that twice the volume of HCl was used for hydrolysis and twice the amount of nonradioactive cystine was added. From one-half of the hydrolysate, cystine was isolated, dried and counted. Since the radioactivity of cystine was very small, the butanol extraction and the precipitation by baryta(9) were omitted. Methionine was also found to have little radioactivity (Exp. A, Table I). The low activities of both cystine and methionine decreased slightly on further recrystallization.

Hydrolysis of liver powder in presence of azo dye. In a preliminary experiment it was found that the dye prepared by coupling tyro-

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‡ We are indebted to Dr. Frances Humm for these analyses.

TABLE I. Distribution of Radioactivity in Fractions of Liver Protein from Rabbit #277.

Exp.	Fraction	Wt of liver protein powder used (g)	Observed radioactivity (counts per min.)	% of total radioactivity (%)
—	Liver protein powder	1.00	4990	(100)
A	Cystine*	3.32	80	.5
	Methionine†	3.32	440	2.7
B	Azodye fraction	1.06	3340	63.1
	Humin‡	1.06	1843	34.9

* Based on 332 mg of added cystine.
† " " 294 " " " methionine.
‡ Dried without washing.

sine with 2 equivalents of diazobenzenesulfonic acid is rather stable in our hydrolysis procedure and that it passes through a Dowex 50 column at pH 2 whereas amino acids are quantitatively adsorbed(10). Approximately 100 mg of the dye, prepared from diazotized sulfanilic acid and tyrosine(11) were added to 1.06 g of the radioactive liver protein. The mixture was hydrolyzed by boiling for 6 hours with 5 *N* HCl. Humin was removed by filtration, dried without further washing and counted. The filtrate was allowed to pass through a Dowex column (height 30 cm, diameter 20 mm) at a pH of 2. The solution flowing through the column was collected in portions of 5 ml. None of these gave a positive ninhydrin reaction. The dye was concentrated in 4 successive fractions. An aliquot of the combined fractions was dried and counted (Exp. B, Table I).

Discussion. Table I demonstrates that the liver, 4 weeks after injection of sulfanilazo-BGG contains approximately 0.9% of the injected radioactivity. Neither the cystine nor the methionine fraction of the liver protein contain significant amounts of the radioactive sulfur. Most of the activity is found in a fraction which, in contrast to amino acids and peptides, passes through a Dowex 50 column at pH 2 and, in this respect behaves like sulfanilic acid, sulfanilazo tyrosine, or similar compounds containing the strongly acidic arylsulfonate ion. Another considerable part of the radioactive material was found in the humin residue; since this had not been washed, part or all of its activity is due to adsorbed azophenylsulfonate derivatives. We have to conclude, therefore, that most of the initially-injected S^{35}-containing azophenylsulfonate groups, 4 weeks after injection, are either unchanged or are converted into metabolic derivatives which still contain the arylsulfonate group. The radioactivity found corresponds to about 2 μg of azophenylsulfonate groups (or 40 μg of the injected antigen) per g of liver protein, an amount too small to be detected spectrophotometrically in the hydrolysate.

Although the radioactive azophenylsulfonate groups found in our experiments are protein-bound, we cannot decide whether they are still bound to BGG. It is quite possible that tyrosine, histidine or peptide residues combined with S^{35}-sulfanilazo groups are detached in the liver from BGG, and by transpeptidation, are transferred to liver proteins. Since BGG in our experiments acts merely as nonspecific carrier and is not responsible for the serological specificity of the azoprotein(4), its fate in the injected animal has not the same significance as that of the determinant *p*-azophenylsulfonate groups.

Summary. S^{35}-sulfanilic acid was diazotized, coupled to beef serum γ-globulin and injected into rabbits. Four weeks after injection the animals were sacrificed. The liver protein, containing approximately 0.9% of the injected radioactivity, was hydrolyzed; cystine and methionine were isolated and found to contain only about 3% of the total radioactivity. Most of the activity was associated with a fraction which on chromatography behaved like an azo dye prepared from tyrosine and diazotized sulfanilic acid. It is concluded that azophenylsulfonate groups persist either unchanged or as slightly changed derivatives in the tissues over periods of more than one month.

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Metabolic Interrelations Between Intrinsic Factor and Vit. B_{12}.* IV. Relative Thermostability of Some Intrinsic Factor Preparations. (21495)

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Thermolability of Castle's intrinsic factor has been considered to be one of its main characteristics. This thermolability was first demonstrated over 20 years ago with the original Castle's technic on patients with pernicious anemia in relapse following oral administration of beef muscle, as source of extrinsic factor, and heated and unheated human gastric juice, as source of intrinsic factor(1,2). Inactivation of the intrinsic factor resulted in abolishment of the usual reticulocyte and red cell responses to this treatment. When vit. B_{12} was identified as extrinsic factor and substituted for beef muscle, a similar inactivation of intrinsic factor was demonstrated, when gastric juice was heated(3). The addition *in vitro* of vit. B_{12} and boiled gastric juice, or boiled or autoclaved mixture of vit. B_{12} with gastric juice, to a megaloblastic bone marrow, caused no maturating effect upon the latter, while addition of unheated gastric juice with B_{12} was highly effective(6). More recently thermolability of intrinsic factor in human gastric juice was demonstrated also with the isotope technic(7). Intrinsic factor-containing extracts from hog stomach were also found to be thermolabile(8-10). However, when the desiccated duodenal hog mucosa was incubated 4 hours with vit. B_{12} at pH 6.8 and 37°C, before the mixture was heated to 100°C for 5 minutes, some hematopoietic activity could be demonstrated by a slight reticulocyte response and clinical improvement(10). Bethell *et al.*(11) showed that when an extract of hog duodenal mucosa was incubated with vit. B_{12}, digested with trypsin for several hours, and then heated to 90-100°C for 10 minutes, the resulting product still possessed a definite hematopoietic activity, as shown by excellent therapeutic response obtained in a patient with pernicious anemia in relapse. Subsequently, Spies *et al.*(12) and Glass and Boyd(13) reported on patients with pernicious anemia in relapse, that when Bifacton, a combination product of intrinsic factor concentrate from hog stomach and vit. B_{12} was boiled in water for 30 minutes, its hematopoietic activity was still preserved.

With the advent of isotope technics and availability of potent intrinsic factor preparations from animal sources, it became possible to re-investigate this problem.

Method. Assay of intrinsic factor activity by hepatic uptake technic(14) was used. The method is based on determination of the increment in radioactivity counts over the liver following oral administration of 0.5 μg of radioactive vit. B_{12} of specific activity of 771 μc/mg or 1-2 μg of radioactive Co^{60}-B_{12} of specific activity of 212 μc/mg, to which the intrinsic factor preparation tested is added. Hepatic and control abdominal counts are taken with the scintillation counter on 2 consecutive days between 6th and 10th day of

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TABLE I. Hepatic Uptake of Radioactivity following Oral Administration of Radioactive Co^{60}-B$_{12}$ Alone and with Boiled or Unboiled Acid Gastric Juice.

Test #	Case #	Preparation given by mouth	Mean uptake of radioactivity calculated in counts/min./1 μc Co^{60} ingested		
			Liver count (LC)	Abdominal control count (AC)	Difference (LC − AC)
		Radioactive vit. B$_{12}$ alone			
1	1	2 μg Co^{60}-B$_{12}$ (.42 μc)	12	5	7
2	2	2 μg ”	45*	148	Neg.
3	2	1 μg ” (.21 μc)	38*	90	”
4	3		0	0	0
5	4		0	0	0
6	5		33*	190	Neg.
7	6		0	248	”
8	6	20 μg ” (2.0 μc)	0	0	0
		Radioactive vit. B$_{12}$ plus *boiled* acid gastric juice of humans			
9	3	1 μg Co^{60}-B$_{12}$ (0.21 μc) + 100 ml boiled acid gastric juice	38*	48	Neg.
10	4	*Idem*	47*	38	9
11	4	.5 μg Co^{60}-B$_{12}$ (.385 μc) as above	0	0	0
		Radioactive vit. B$_{12}$ plus *unboiled* acid gastric juice of humans			
12	1	2 μg Co^{60}-B$_{12}$ (.42 μc) + 75 ml native acid gastric juice	357	181	176
13	2	*Idem*	264	31	233
14	7	1 μg Co^{60}-B$_{12}$ (.21 μc) + lyophilized powder of 100 ml acid gastric juice	580	38	542
15	3	*Idem*, but 121 ml acid gastric juice	195	105	90
16	2	” but 98 ml acid gastric juice	467	243	224

* In view of high abdominal counts exceeding hepatic counts, this figure mainly represents the scattered radiation from radioactive material retained in intestine, and should not be interpreted as hepatic uptake.

experimental period in direct contact with the skin over 3 projections of liver, and 3 at the lower, mid and left abdomen, as described in previous communications(14-16). We used this method on patients with pernicious anemia in remission for measurements of the intrinsic factor activity of preparations tested and showed that it compares favorably with the classical method of hematopoietic responses on patients with pernicious anemia in relapse(17). The present studies were made on 7 patients with pernicious anemia in remission. Diagnosis of the disease was ascertained when patients were in the period of full blown relapse by all clinical and hematological criteria. These included: macrocytic anemia, megaloblastic bone marrow, gastric achylia, signs of combined or posterior degeneration of the cord, as well as positive therapeutic response either to parenteral treatment with vit. B$_{12}$, or to oral treatment with vit. B$_{12}$ in association with a potent intrinsic factor concentrate from hog stomach (13). The pooled human gastric juice was obtained after histamine or insulin stimulation from several individuals with gastric hyperacidity or duodenal ulcer(18). One part of the collected juice was filtered and before its use stored under refrigeration at 4-6°C for a few weeks without neutralization. Another was dialyzed extensively in electrically stirred dialyzers immediately after collection and freeze-dried in Stokes lyophilizer. The highly active intrinsic factor preparations from hog stomach (not containing vit. B$_{12}$) were supplied through the courtesy of Dr. Kenneth W. Thompson from Organon.

Results. Human gastric juice. When 1-20 μg radioactive vit. B$_{12}$ was given alone to 6 patients with pernicious anemia in remission (8 tests), no hepatic uptake was observed. When 75 ml pooled native gastric juice, or lyophilized powder derived from 98-121 ml of gastric juice was added to the same dose of radioactive vit. B$_{12}$ and given to 4 patients with pernicious anemia (5 tests), a positive

TABLE II. Hepatic Uptake of Radioactivity following Oral Administration of 1 μg Co^{60}-B_{12} Containing 0.21 μc Co^{60} Together with Boiled or Unboiled Intrinsic Factor Concentrate from Hog Stomach.

Test #	Case #	Dose and No. of intrinsic factor concentrate	Treatment of I. F. concentrate	Hepatic uptake of radioactivity in cpm/1 μc Co^{60} ingested
		mg		
17	2	58 IF 4635	Unboiled	505
18	3	58	"	657
19	1	58	"	371
20	2	5 I-M-32	"	257
21	2	58 IF 4635	Boiled in 0.1N HCl	0
22	3	58	*Idem*	0
23	2	58	Boiled in water	281
24	3	58	*Idem*	210
25	2	3.7 I-M-32	"	405

hepatic uptake of radioactivity was obtained, which amounted to 195 to 580 counts per minute above background (calculated per 1 μc Co^{60} ingested) (Table I). However, when radioactive vit. B_{12} was given to 2 of these patients (3 tests) with 100 ml of acid gastric juice (pH 1.2-1.4) which was boiled for 30 minutes, no uptake of radioactivity by the liver was observed, or only in traces, the hepatic counts being similar to abdominal control counts (Table I).

Intrinsic factor concentrate IF #4635. These studies were done on 4 patients with pernicious anemia in remission, and results are listed in Table II. To 3 of these patients we gave 1 μg Co^{60}-B_{12} together with 58 mg of the intrinsic factor concentrate #4635, Organon, a dose equivalent in potency to that contained in 1 USP unit of Bifacton. In each instance a positive hepatic uptake of radioactivity was observed which ranged from 371 to 657 counts per minute above background (calculated per 1 μc Co^{60} ingested). However, when the same intrinsic factor concentrate was dissolved in 30 ml of 1/10 normal HCl (final pH of the mixture about 1.2), boiled for 30 minutes and given with 1 μg Co^{60}-B_{12} to 2 of these patients no uptake of radioactive vit. B_{12} by the liver could be demonstrated. This indicates destruction or inactivation of the intrinsic factor activity of this preparation by boiling at an acid pH. We obtained similar results in a third patient with pernicious anemia (not listed in Table II) to whom we previously gave 15 μg of radioactive vit. B_{12} containing 0.42 μc of radioactive Co^{60} together with 58 mg of the same intrinsic factor concentrate, and in whom a positive hepatic uptake of 83 counts per minute was obtained. The administration of the same preparation after boiling for 30 minutes in 1/10 normal HCl, with 15 μg of radioactive B_{12} resulted in 6 times lesser uptake of radioactivity by the liver (14 counts per minute). In subsequent studies we suspended the same intrinsic factor concentrate in 30 ml of the distilled water (final pH 5.6-6.0). After homogenization the suspension was boiled for one-half hour, and given in 2 tests to the same 2 patients with pernicious anemia in remission together with 1 μg of radioactive vit. B_{12}. A positive uptake of radioactivity by the liver was observed in both instances. This was from 210 to 281 counts per minute above background, calculated per 1 μc Co^{60} ingested, and highly exceeded the averaged control counts over the abdomen. The counts obtained represented 56 respectively 32% of the hepatic uptake noted when the same intrinsic factor preparation was given to the same patients without being boiled.

Fraction I-M-32 of the intrinsic factor concentrate[†]. 5 mg were given with 1 μg of radioactive vit. B_{12} to a patient with pernicious anemia in remission, and a positive uptake of 257 counts per minute above background was noted; this exceeded the control abdominal counts several times, indicative of definite intrinsic factor potency of this preparation. The same fraction was then boiled in distilled water (pH 6) for 30 minutes and 3.7 mg of it with the same dose of radioactive vit. B_{12} was given to the same patient. A definite positive hepatic uptake was obtained which even exceeded that observed after administration of 5 mg of the unboiled preparation (Table II).

[†] This fraction was obtained from intrinsic factor concentrate of the hog stomach by means of preparative electrophoresis by Dr. Kurt G. Stern from Brooklyn Polytechnic Institute, and supplied to us through the courtesy of Dr. Kenneth W. Thompson of Organon.

Discussion. The results of the isotope assay of intrinsic factor activity of the acid (non-neutralized) gastric juice of humans are in line with earlier data obtained with other methods on the neutralized gastric juice, namely, that boiling of the human gastric juice at an acid pH destroys or inactivates intrinsic factor contained in it(1-5).

Our data indicate, however, that the thermolability of the hog stomach extracts is a relative one. It can be demonstrated when the preparation is boiled with 1/10 normal HCl at pH of about 1.2. When the preparation is, however, boiled at a neutral pH (about 6) the intrinsic factor activity becomes only partly impaired (IF 4635) or not modified at all (fraction I-M-32).

The explanation for the relative thermostability of some of the intrinsic factor preparations of animal origin when they are boiled at neutral pH, and their thermolability when boiled with HCl at pH 1.2 is unknown to us. Perhaps the oxidation of the material, when boiled with HCl, is the main reason for the destruction or inactivation of the intrinsic factor. It might also be that the greater solubility of the intrinsic factor concentrate in acid than in water contributes to easier destruction of the intrinsic factor activity on boiling. Finally, it is possible that intrinsic factor of the animal stomach is more thermostable to heat at neutral pH than at acid pH, similar to the thermostability of the vit. B_{12} binding substance from the human gastric juice at a neutral pH and its thermolability at the acid pH(5). The intrinsic factor contained in mucosal extracts and concentrates from animal stomach appears to be better protected against heat at neutral pH than at the acid one, but this does not necessarily mean, however, that the intrinsic factor from the hog stomach is different from that of man. At any rate, the earlier conclusion(11) in regard to animal preparations, namely, that "intrinsic factor is relatively heat stable in combination with cobalamine" and to which others(12) and we as well(13) have previously subscribed, should be amplified in so far as some of the intrinsic factor concentrates from animal stomach are relatively thermostable at neutral pH, even if they are heated without prior combining with B_{12}. This relative thermostability may depend on some protection given to intrinsic factor by other proteins which are highly concentrated in these preparations during the manufacturing process.

Conclusions. An assay of the intrinsic factor activity of native, acid gastric juices of humans by means of the scintillation measurements of the hepatic uptake of radioactive vit. B_{12} on patients with pernicious anemia in remission, has confirmed the thermolability of the human intrinsic factor and its destruction or inactivation by boiling at pH 1.2-1.4 for 30 minutes. Similar studies done on intrinsic factor concentrates from hog stomach revealed inactivation or destruction of its intrinsic factor activity by boiling in 1/10 normal HCl (pH about 1.2) for 30 minutes, but a relative heat stability of the same preparations, when boiled in water at a neutral pH of about 6.0. Therefore, the thermostability of some of the "intrinsic factor concentrates with vit. B_{12}" from hog stomach, when boiled in water, is apparently not due to the prior combination with vit. B_{12} because it can also be demonstrated on concentrates boiled in water prior to addition of the vitamin.

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Heparin-Activated Enzyme System of Human Plasma.* (21496)

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Recently, it was demonstrated that incubation of egg lipoprotein or human S_f 20-400 lipoprotein with plasma of a human given an intravenous injection of 100 mg sodium heparin (Lederle) results in hydrolysis of the lipoprotein triglycerides with concomitant release of fatty acids(1-3). The increase in acidity of the incubation mixture was demonstrated by infrared analysis of a lipid extract separated into various components by a modification of the chromatographic technics developed by Borgstrom(4). The release of fatty acid was confirmed subsequently by others(5,6). In this communication we wish to present additional data on the subject. The effects described were not observed when either serum to which heparin had been added *in vitro* or blood drawn into heparinized vessels was incubated with a lipoprotein substrate. Plasma obtained from a human subject 15-30 minutes after administration of heparin was in every case capable of bringing about liberation of fatty acid from suitable substrates.

Materials and methods. Pre-heparin serum, post-heparin plasma, and egg lipoprotein were obtained by methods described in the previous communication(3); human S_f 20-400 lipoproteins were obtained by the method of Lindgren, Elliott, and Gofman(7). In general, 1-2 ml of diluted egg lipoprotein (elp) or undiluted human lipoprotein was incubated with 2 or 3 ml serum or plasma at 37°C for periods ranging from 3 to 8 hours. The incubations were performed with parallel sets of samples, one set containing pre-heparin serum, the other post-heparin plasma from the same individual. Extraction of lipids from the incubation mixture was accomplished by the methanol-ethyl ether procedure indicated previously(3). In analyzing the total lipid extract from an incubation mixture to determine triglyceride, fatty acid, and in some instances other lipids as well, a lipid separation was carried out by means of a silicic acid adsorption column in the manner described by Borgstrom(4) and discussed in the previous communication(3). After their separation, triglyceride and fatty acid were usually determined by 2-component infrared spectrophotometric analysis. The Beer-Lambert law may be written in a generalized form for i components: $A = l\sum_i a_i c_i$, where A is the observed optical density (absorbance), l the cell length, a the absorptivity of component i, and c the concentration of component i. Measurement of optical density at 5.8 and 5.9 μ determined fatty acid and triglyceride. In general, the infrared measurements themselves were the same as mentioned previously(3,8). In some

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TABLE I. Release of Fatty Acid from Lipoprotein Triglycerides after Incubation with Pre-heparin Serum or Post-heparin Plasma and Various Compounds at 37°C for 4 Hours.

Incubation constituents	mg fatty acid increase	mg triglyceride decrease*
1), 2 ml elp†	12.8, 12.5, 12.5	13.6, 13.9, 13.6
2), *Idem* , 0.02 mg s.h.‡	0, .3, .3	0, .2, .3
2), ” §	.4, .5, .1	.4, .3, .3
1), ” , DFP (1×10^{-4} M)‖	.4, .5, .7	.4, .5, .6
2), ” , ”	.4, .3, .2	.5, .3, .2
1), ” , oxygen	12.3, 12.4, 12.9	13.7, 13.6, 14.1
2), ” , ”	.2	.3
1), ” , nitrogen	13.4, 13.5	14.7, 14.5
2), ” , ”	.4	.2
1), ” , α-naphthol (1×10^{-3} M)	12.7, 12.9	13.6, 13.8
2), ” , *Idem*	.3	.4
1), ” , p-chloromercuribenzoate (1×10^{-3} M)¶	**	13.5, 13.7, 15.1
2), ” , *Idem* ¶	**	.4
1), ” , o-iodosobenzoate (1×10^{-3} M)¶	**	13.4, 13.8, 13.7
2), ” , *Idem* ¶	**	.3
1), ” , iodoacetate (1×10^{-3} M)¶	**	13.4, 13.9, 13.7
2), ” , *Idem* ¶	**	.2
1), 1 ml elp	8.2, 8.4, 8.3	9.1, 9.2, 9.5
2), *Idem*	.2, .1	.2, .3
1), ” , cysteine (8×10^{-3} M)	8.3, 8.5, 8.5	9.0, 9.2, 9.5
2), ” , *Idem*	.2, .4	0, .4
1), ” , ” , ATP (1×10^{-3} M), CoA (1×10^{-3} M)	7.3, 7.5, 7.7	8.4, 8.5, 9.0
2), 1 ml elp, cysteine (8×10^{-3} M), ATP (1×10^{-3} M), CoA (1×10^{-3} M)	.2, .3	.4, .4
1), 1 ml elp, cysteine, ATP (1×10^{-3} M)	7.9, 8.1	8.6, 9.0
2), *Idem* , *Idem*	.3, .4	.2, .4
1), ” , CoA (1×10^{-3} M)	8.0, 8.0	8.8, 8.7
2), *Idem* , *Idem*	.3, .4	.1, .3

* No significant decrease in phospholipids or cholesteryl esters. First values listed in columns of triglyceride decrease and fatty acid increase obtained in the same experiment, the second values in the same experiment, and so on.

† 1) = 3 ml post-heparin plasma; elp = egg lipoprotein.

‡ 2) = 3 ml pre-heparin serum; s.h. = sodium heparin and present in all incubations with 2).

§ Pre-heparin blood drawn into heparinized syringe.

‖ Incubated with serum or plasma for 20 min. at 25°C before addition to elp.

¶ ” ” ” ” ” ” 120 ” ” ” ” ” ” ”.

** Data not reliable.

cases, the fatty acids were separated from triglycerides by extracting the fatty acids with alkaline 50% ethanol-water and reextraction of the acids after acidification(4). The acids were then titrated or their infrared absorption at 5.9 μ measured.

Results. Action of post-heparin plasma on S_f 6 lipoprotein. Since the triglycerides of human S_f 20-400 lipoproteins are extremely susceptible to hydrolysis with accompanying fatty acid release, it seemed of interest to see whether the human S_f 6 lipoprotein, of triglyceride content 10% of its total lipid, could serve as a substrate for the post-heparin plasma. A sample of S_f 6 lipoprotein, prepared according to previously published methods(7), that contained 20.2 mg triglyceride was incubated for 8 hours at 37.5°C with 3 ml post-heparin plasma. The amount of fatty acid found after incubation (1 mg) agreed closely with that present in the system before incubation (0.9 mg); incubation of human S_f 20-400 lipoprotein containing 22.0 mg triglyceride with the same plasma resulted in the release of 5.4 mg fatty acid.

Effect of heparin injection on serum tributyrinase activity. That serum possesses lipolytic activity towards tributyrin has been noted by many workers(9). Consequently comparison was made between the tributyrinase activity of serum and that of post-heparin plasma. In experiments carried out by the method of Alper(9), with an incubation time of 2 hours, 3.74, 3.70, and 3.79 ml 0.05 N sodium hydroxide were required to titrate the fatty acids liberated as a result of the action of post-heparin plasma; 3.60, 3.54, and 3,59 ml were required in the incubation with pre-heparin serum.

Inhibition of lipolysis by fatty acid. Since the plasma of an individual who had eaten a very lipemic breakfast before heparin injection even though warned against this practice contained a considerable amount of fatty acid, experiments were performed to investigate the role of free fatty acid in the triglyceride lipolysis. After incubation of 1 ml elp with 3 ml post-heparin plasma for 4 hours at 37°C, the initial level in the system before incubation of 9.5 mg fatty acid (of which 90% was contributed by the plasma) rose to 10.4 mg after 4 hours and 11.0 mg after 6 hours. Plasma from a fasting individual, after it had been incubated with the same substrate, produced a 6 mg rise after 4 hours. Addition of fatty acid *in vitro* to an incubation mixture also inhibits fatty acid release from lipoprotein substrates(10).

Effects of various substances on fatty acid release. The effects of such substances as adenosine triphosphate (ATP) and Coenzyme A (CoA), diisopropylfluorophosphate (DFP), aerobiosis, anaerobiosis (with nitrogen), α-naphthol, p-chloromercuribenzoate, o-iodosobenzoate, and iodoacetate on fatty acid release from lipoprotein triglycerides are indicated in Table I.

Discussion. Aldridge(11) has shown that the esterases of serum may be classified into 2 distinct types (A and B) by their sensitivity to organophosphorus inhibitors, the B type esterase being inhibited by low concentrations and the A-type esterase being resistant. Our data indicate DFP (diisopropylfluorophosphate) to be extremely effective in preventing fatty acid release from triglycerides, and this sensitivity to DFP suggests that the heparin-activated enzyme is a B-type esterase.

Summary. 1. In distinction to the S_f 20-400 lipoproteins, the S_f 6 lipoprotein of the human is not a good substrate for fatty acid release by the heparin-activated enzyme system of plasma. 2. The serum tributyrinase activity is not enhanced significantly after heparin injection. 3. The lipolysis of the lipoprotein triglycerides is significantly inhibited by fatty acid itself. 4. The fatty acid release resulting from the incubation of suitable lipoproteins with post-heparin plasma is unaffected by oxygen, the antioxidant α-naphthol, and sulfhydryl inhibitors; anaerobiosis with nitrogen produces at most a 10% increase in fatty acid release. Fatty acid release is inhibited at most 10% by ATP and Co A, and at most 5% by either compound. The inhibition by DFP suggests the enzyme to be an esterase.

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Effect of 'Insulinase-Inhibitor' on Destruction of Insulin by Intact Mouse.[*][†]

(21497)

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Insulin is destroyed *in vitro* by homogenates, extracts and slices of liver and other tissues(1,2,3) and *in vivo* by the intact mouse (4). This inactivation and destruction of insulin appears to be due to the action of an enzyme system, insulinase, which is relatively specific in catalyzing the hydrolysis of insulin (5).

During the course of studies on the purification of insulinase it was observed that a non-protein fraction of liver contained a substance which inhibited the *in vitro* activity of insulinase but was without effect on the proteolytic activity of various proteolytic enzymes and liver extracts(6). This factor, 'insulinase-inhibitor', was found to be quite soluble in water, acid stable, heat stable, alkali unstable, freely dialysable and freely diffusible into slices of liver(2). In order to determine whether insulinase-inhibitor is effective *in vivo* as well as *in vitro*, the effect of crude insulinase-inhibitor preparations on the destruction of insulin by the intact mouse was studied.

Method. Fed, male mice of from 18 to 25 g in weight were given an intraperitoneal injection of 0.02 ml of a solution of insulin per gram and a subcutaneous injection of 1 ml of a 5.5% glucose solution. The insulin consisted of a mixture of crystalline zinc insulin[‡] and tracer quantities of I^{131} labeled insulin prepared by the Abbott Laboratories. At various intervals before the injection of insulin, mice were given a subcutaneous injection of a solution of insulinase-inhibitor while a similar number of mice were given a similar volume of saline. Each mouse was kept in an individual beaker. At 60 minutes after the injection of insulin, each mouse was cut up with shears into a Waring Blendor and homogenized with 3 volumes of 10% trichloracetic acid. The contents of each animal's beaker were washed into the Blendor with the trichloracetic acid. Two 20 ml aliquots of the homogenate were poured into tared 50 ml plastic centrifuge tubes and the weight of each aliquot was determined. The remainder of the mixture was filtered and two 20 ml aliquots of the filtrate were weighed in tared tubes. The radioactivity of the aliquots was then measured in a well-type gamma counter. All counts were corrected for background and coincidence. The percentage of the total counts present in the trichloracetic acid filtrate was computed and served as an index of the fraction of the administered insulin which was destroyed by each mouse in 60 minutes(4). The insulinase-inhibitor employed in this study was as effective *in vitro* as the preparation employed in previous studies(6) but was prepared as follows: 100 g of fresh beef liver were homogenized with 1000 ml water and the pH adjusted to 4.8 with hydrochloric acid. The homogenate was boiled for 10 minutes and filtered. The filtrate was dried by lyophilization. The lyophilized product was extracted with 20 volumes of glacial acetic acid and the acetic acid filtrate was precipitated with 3 volumes of cold acetone. The precipitate was washed with acetone and dried with ethyl ether. The dried precipitate was dissolved in water and

TABLE I. Influence of Interval between Insulinase-Inhibitor and Insulin Injections on Destruction of Insulin by Intact Mice. Mean ± S.E. of the % of administered I^{131} found in the non-protein fraction of homogenates of mice. All mice given subcutaneous injection of 4 mg inhibitor/100 g and intraperitoneal injection of 40 U I^{131} labeled insulin/100 g.

No inhibitor	Inhibitor—min. before insulin			
	0	15	30	60
32.4 ± 2.03	22.4 ± .71	16.12 ± 2.37	15.52 ± 1.66	13.67 ± .63

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[†] Presented in part before the Am. Chem. Soc., New York City, Sept., 1954.

[‡] We are indebted to Eli Lilly Co. for generous supplies of crystalline insulin which assayed 25 units per mg.

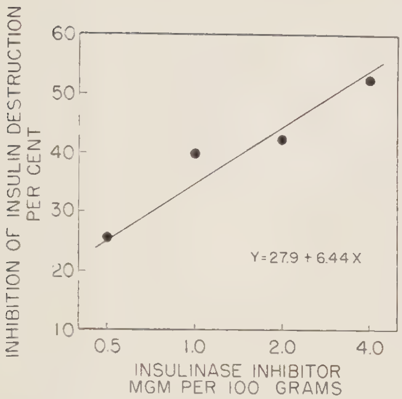

FIG. 1. Effect of different quantities of 'insulinase-inhibitor' on destruction of exogenous insulin by the mouse. Semilogarithmic plot of % inhibition of insulin destruction computed from the difference between animals given the same quantity of insulin 30 min. after subcutaneous injection of saline or inhibitor. Each point represents mean of 5 mice.

adjusted to pH 7.0. A 20% solution of the inhibitor was utilized for injections.

Results. In a previous study(3) it was demonstrated that the degradation of I^{131} labeled insulin, as measured by the release of I^{131} into the trichloracetic acid soluble fraction parallels the inactivation of insulin. The efficiency of the procedure utilized in this study has been reported in another communication(4). In that report it was also demonstrated that approximately 40 units of insulin per 100 g body weight is necessary to saturate the system responsible for the destruction of insulin in the intact mouse. Accordingly, the effect of insulinase-inhibitor was studied in animals which were given 40 units insulin per 100 g body weight.

Preliminary experiments established that the maximal effect of the injection of insulinase-inhibitor occurs in animals which are given the inhibitor in from 15 to 60 minutes before the injection of insulin (Table I). Consequently, the effect of different quantities of inhibitor was determined in mice which received the inhibitor 30 minutes before the administration of the insulin.

The effect of different quantities of insulinase-inhibitor is illustrated in Fig. 1 in which the decrease in the rate of insulin degradation by the inhibitor treated mice is expressed as per cent inhibition of the insulin destruction observed in the saline injected mice. It is apparent that the subcutaneous injection of even 0.5 mg of the crude inhibitor preparation per gram of mouse results in a significant inhibition of the destruction of exogenous insulin by the intact mouse. The relationship between the degree of inhibition of insulin destruction and the quantity of inhibitor injected is indicated by the significant ($P<0.01$) regression of the per cent of inhibition on the logarithm of the quantity of inhibitor administered.

The influence of different routes of administration on the effectiveness of the inhibitor was determined. One group of 3 mice was given a subcutaneous injection of 4 mg inhibitor per gram in a 20% solution. A second group of mice was given an intraperitoneal injection of a similar quantity of inhibitor. A third group was given a subcutaneous injection of a similar volume of 0.85% sodium chloride. Although the group which was given the subcutaneous injection showed a diminution of 56.1% in the rate of insulin destruction, the group which was given the inhibitor intraperitoneally showed a diminution of 77.7%, which is significantly greater than the aforementioned.

To determine whether or not insulinase-inhibitor behaves like a competitive inhibitor in the intact animal, groups of 3 mice were given 2.0, 4.0 and 40 units of insulin per 100 g after a preliminary subcutaneous injection with saline or with 4 mg of inhibitor per gram of mouse. The quantity of insulin degraded by each mouse was computed from the I^{131} released into the non-protein fraction. A Lineweaver-Burk(7) plot of the data (Fig. 2) suggests that the inhibitor acts as a competitive inhibitor.

Discussion. In accord with the observation on the inhibition of insulin inactivation and destruction by extracts and slices of liver (2,6), insulinase-inhibitor is effective also in decreasing the destruction of exogenous insulin by the intact mouse. Similar effects have been noted with much purer preparations from liver as well as from other sources such as corn gluten and partial hydrolysates of various proteins(8).

Attempts to demonstrate an effect of in-

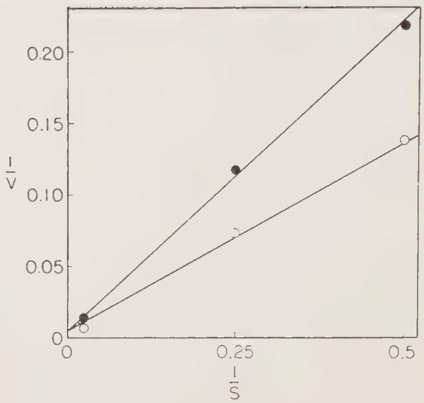

FIG. 2. Effect of 'insulinase-inhibitor' on destruction of exogenous insulin by mice given various quantities of I^{131} labeled insulin. All animals received subcutaneous injection of saline (○) or inhibitor (●) 30 min. before injection of I^{131} labeled insulin. Each point represents the mean of 3 mice. V = units I^{131} labeled insulin destroyed/100 g of mouse in 60 min. computed from % of total I^{131} found in non-protein fraction. S = units I^{131} insulin injected intraperitoneally/100 g of mouse.

sulinase-inhibitor on the blood sugar of normal animals have failed. This failure may be attributed to the fact that with a rate of destruction of approximately 30% of the available insulin per hour(4), the quantity of endogenous insulin undergoing such destruction would be extremely small because the concentration of available endogenous insulin is relatively very low at any one moment.

Unpublished studies on the action of insulinase-inhibitor on the degradation of insulin by extracts of liver indicate that it is a competitive inhibitor(8). One preparation which was utilized in a study on the inhibition of the destruction of insulin by liver slices was found to be a non-competitive inhibitor (2). Subsequently, it was discovered that that preparation was made from a supply of dried liver which was obtained commercially and which was contaminated with relatively large quantities of copper. The inhibitor utilized in the present study was prepared from fresh beef liver and after ashing it was ineffective *in vitro*. The identity of insulinase-inhibitor remains unknown other than it is a peptide (or peptides) of low molecular weight which is resistant to hydrolysis by acid, trypsin and pepsin and which resembles strepogenin(9) in a number of characteristics.

It has been proposed that the insulin insufficiency of diabetes mellitus may be due to an increase in insulinase activity(10). Such an increase may be due to a spontaneous increase in the activity of insulinase or to a decrease in the availability of insulinase-inhibitor. The present study supports the possibility that insulinase-inhibitor may influence the activity of insulinase *in vivo*. Whether or not the inhibitor is effective in the treatment of patients with diabetes mellitus must await its purification and clinical trial.

Summary. A non-protein fraction of liver which can inhibit the destruction of insulin by extracts and slices of liver, is effective also in inhibiting the destruction of exogenous insulin by the intact mouse. This factor, insulinase-inhibitor, appears to act as a competitive inhibitor.

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Inhibition of Multiplication of Lee Influenza Virus by Canavanine.*†‡ (21498)

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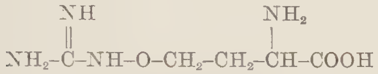
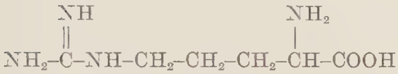
L-Canavanine, an amino acid occurring in jack beans, is a close structural analogue of arginine. The degree of similarity is best appreciated by inspection of their formulae.



$$NH_2-\overset{\overset{NH}{\|}}{C}-NH-O-CH_2-CH_2-\overset{\overset{NH_2}{|}}{CH}-COOH$$

Canavanine



$$NH_2-\overset{\overset{NH}{\|}}{C}-NH-CH_2-CH_2-CH_2-\overset{\overset{NH_2}{|}}{CH}-COOH$$

Arginine

This uncommon amino acid has been found to be a potent growth inhibitor for *Neurospora*, for certain species of lactobacilli, and for *Escherichia coli*(1,2). Furthermore, this inhibition of growth has been shown to be based on competitive antagonism of arginine; it was reversible by the latter amino acid and in most cases a high degree of specificity was observed.

Eaton(3) found the basic amino acids arginine, lysine, and ornithine inhibitory to the influenza and mumps viruses cultivated *in vitro* when these amino acids were present in concentrations of 5 to 10 mg per ml. However Knight(11) has shown that both arginine and lysine are present in purified preparations of Lee influenza virus. Apparently arginine is utilized in biosynthesis of the virus, but if present in sufficient concentration in virus tissue cultures may antagonize some other essential reaction. While the present study was in progress Pearson, Lagerborg and Winzler(4) reported on the effects of a large number of amino acids and related compounds on the growth of the mouse encephalomyelitis virus in tissue culture. L-lysine and fluorophenylalanine were among the most effective inhibitors and canavanine was also found to be inhibitory.

Methods. For experiments with intact chick embryos, the Lee influenza virus obtained from the American Type Culture Collection was employed. It was used in the form of allantoic fluid, either fresh or lyophilized. In either case infectivity titrations were conducted in chick embryos and the dosage of virus to be employed was calculated from the titration data. Nine- or 10-day-old chick embryos were used in all experiments. The canavanine was obtained from jack bean meal by ethanol extraction and precipitation as the flavianate, according to the method of Horowitz and Fling(5). The final preparation was in the form of canavanine sulfate or in some cases the free base. The latter was preferable as it did not change the pH of allantoic fluids of treated chick embryos. However, it was somewhat unstable and decomposed very slowly even when held at –10°C in an anhydrous condition. The sulfate in a dose of 20 mg reduced the pH from about 8.3 to about 7.8. For use in chick embryo experiments either compound was dissolved in distilled water to give a concentration of 100 mg/ml and sterilized by filtration through an ultrafine fritted glass filter. Where the effect of the compound upon growth of the virus in chick embryo was to be studied, the sterile solution of the compound was injected intra-allantoically into embryos in groups of 10 or more, and after an hour the desired dose of virus was injected by the same route. In some cases the compound was administered at various intervals after the virus. A similar group

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[†] We are much indebted to Mr. J. A. Hanson of the J. A. Hanson Leghorn Farm in Corvallis, Oregon, who generously contributed large numbers of hatching eggs.

[‡] Published with the approval of Publications Committee of Oregon State College as research paper no. 264, School of Science, Dept. of Bacteriology.

TABLE I. Inhibitory Effect of Canavanine on Growth of the Lee Influenza Virus in the Chick Embryo.

Dosage of canavanine per egg, mg	No. eggs tested	% of eggs showing hemagglutinin titers of 0	(20-80)	(120-320)	(480 and above)	Geometric mean of hemagglutinin titers* of pooled fluids
20	83	65.0	24.1	10.9	0	7.7
10	45	28.9	24.4	40.0	6.7	81.9
5	34	41.0	29.5	20.6	8.9	116.5
2	20	25.0	20.0	45.0	10.0	138.6
0 Controls	108	10.2	20.4	48.2	21.2	250.4

Table summarizes data of 11 experiments in which canavanine was injected in allantoic sac, followed in about an hour by 50 EID_{50} of virus by same route. After 44 hr incubation at 35°C hemagglutinin titers of each living egg and of the pooled allantoic fluids of all eggs in each group were determined.

* Expressed as reciprocal of highest dilution of allantoic fluid giving complete 4+ hemagglutination.

of control embryos received virus only, or virus plus sterile distilled water. All embryos were incubated at 35°C for 44 hours, and any dead embryos discarded. Living embryos were then chilled at –20°C for an hour. The allantoic fluids were harvested and titrated individually for virus content. In most cases the hemagglutinating activity for washed chicken erythrocytes was determined, using the pattern method described by Salk(6). In some cases, however, the fluids from all embryos in the same group were pooled and the virus content measured by infectivity titration in chick embryos. Fifty per cent endpoints were then calculated by the method of Reed and Muench(7). Aseptic precautions were used in all operations and all materials used in each experiment were tested for bacterial contamination by inoculating small samples in thioglycollate broth.

Where experiments were carried out in tissue cultures, the technic employed was essentially that described by Tamm, Folkers and Horsfall(8). In this method small portions of chorioallantoic membrane (wet weight between 130 and 170 mg) from 9- to 11-day chick embryos are suspended in nutrient fluid in 25 x 150 mm Pyrex tubes, closed with rubber stoppers. One piece of tissue and a 2 ml volume of nutrient fluid are added to each tube. The fluid is a buffered glucosol solution of pH 7.28 as described in the above reference. Ten units of penicillin per ml and 40 μg of streptomycin per ml are added. In tissue cultures, the Lee virus was a strain kindly supplied by Dr. Frank L. Horsfall of the Rockefeller Institute. It had become thoroughly adapted to the chick embryo by numerous passages in that organism. In most experiments it was employed in the form of a 1:10 dilution of freshly harvested allantoic fluid virus. Tissue cultures were inoculated with 0.1 ml of this dilution. The resulting virus concentration in the nutrient fluid was never great enough to give significant agglutination of chicken red cells when the fluid was tested at a dilution of 1:10, although in some cases agglutination occurred at a 1:2 dilution. After inoculation, tissue cultures were incubated at 35°C for 44 to 48 hours on a reciprocating shaker having a rate of 90 strokes per minute and a stroke length of 8 cm. The virus content of each culture was then determined by hemagglutinin titration. When canavanine was employed in tissue cultures, it was incorporated in the nutrient fluid at the desired concentration and the latter was sterilized by filtration before the cultures were set up. In the concentrations used it did not produce significant changes in the pH of the buffered glucosol.

Results. The most extensive studies of the effect of canavanine on development of the Lee influenza virus have been carried out in the intact chick embryo. In the majority of experiments the desired dose of the compound was injected into the allantoic cavity of 10 day embryos about an hour before the injection of 50 EID_{50}§ of virus by the same route. After 44 hours of incubation at 35°C the virus content of the allantoic fluids of individual eggs and of the pooled fluids of all eggs in an experimental group was determined by hemagglutinin titration. A summary of the results of most of these experiments is presented in Table I.

It will be noted that a dose of 20 mg of canavanine per egg, which is about the maximum tolerated dose, produced a marked degree of inhibition of the virus, as measured by hemagglutinin titration. Smaller doses of the compound produced correspondingly lesser degrees of inhibition. Two mg per egg, the smallest dose tested, still gave a slight though definite inhibition, as reflected in the mean hemagglutinin titers of the pooled fluids. The difference in this case appears in the table as slightly less than 2 fold when the mean titers of pooled fluids are compared. However, when the titers from individual eggs in the experiments employing the 2 mg dose were examined by means of "Student's" "t" test for comparison of 2 means the difference was found to be significant‖¶(9). It seems apparent, however, that this small dose is approaching the minimum for which a significant effect might be demonstrated. The degree of inhibition observed in individual experiments showed considerable variation. With a 20 mg dose of canavanine and a virus inoculum of 50 EID_{50}, this ranged from complete suppression of hemagglutinin development in some experiments to a 3 or 4 fold reduction in hemagglutinin titer in others. This variation was probably due to some variable factor in different groups of embryonated eggs as it was not encountered in similar experiments employing tissue cultures.

Inhibition of virus development could also be demonstrated when the virus content of the pooled allantoic fluids of canavanine treated and control eggs was determined by means of infectivity titration in chick embryos. Under the experimental conditions outlined above, the virus titer of fluids of canavanine treated eggs was found in several experiments to be from 1 to 3 log units lower than that of untreated controls. It was found that similar results were obtained when the canavanine was injected into the yolk sac of chick embryos about an hour before the injection of 50 EID_{50} of virus into the allantoic sac. The degree of inhibition observed was comparable to that noted when both injections were made into the allantoic sac. The effectiveness of injection into the yolk sac indicated that it was not necessary for the compound to come in direct contact with the inoculated virus in the allantoic sac to produce the inhibitory effect, and suggested that the latter was not due to a direct action of canavanine on the virus.

The influence of the dose of virus in the inoculum on the inhibition of the virus was also investigated. In all such experiments a dose of 20 mg of canavanine was injected in the allantoic sac of groups of embryos about an hour before the desired dose of virus. A range of virus dosage from approximately 10 to 100,000 EID_{50} was explored. As might be expected, the degree of inhibition of the virus, as judged by hemagglutinin titration, was less when large virus inocula were employed. With inocula of less than 100 EID_{50} there was usually at least a 30 fold difference in the hemagglutinin titers of the pooled fluids from treated and untreated embryos. With inocula of 400 up to about 100,000 EID_{50}, this difference varied from 2 to 10 fold in different experiments, and showed no consistent relationship to the virus dosage within this range. It should be noted that in a majority of the experiments reported, observations on the virus content of the allantoic fluids was made

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§ EID_{50} is the dose of virus required to infect half of a group of 6 or more embryos.

‖ For a "t" value of 2.69, P was less than 0.02.

¶ It should be noted that the mean values given in Table I are geometric means. This type of mean has been considered appropriatte in the case of data in which the individual values are changing at a uniform rate rather than by a uniform amount. The application to hemagglutinin titers has been made by Tamm, Folkers and Horsfall(8,12). However, for statistical analysis the logs of the individual titers and the arithmetic mean of these log values were employed for both treated and control groups. The use of various transformations of data, such as the above log transformation is discussed by Bartlett(13). The chief purpose in using the log transformation here is to provide a series of measurements approximating the normal distribution more closely than the data in its original form.

after an incubation period of about 44 hours. It was not surprising to find that the duration of the inhibitory effect following a single dose of the compound was limited. When the incubation period was extended to 68 hours in one experiment the difference in virus content between treated and control eggs was no longer significant. The disposition of the compound in the allantoic sac during this period is unknown.

An effort was made to shed as much light as possible on the mechanism by which the multiplication of the virus was inhibited by canavanine. One approach was to examine the effect of varying the time interval between injection of the virus and the compound in the allantoic sac. Accordingly, comparisons were made between different groups of embryos treated with 20 mg of canavanine either an hour before the injection of 50 EID_{50} of virus, or at intervals of 2 hours or 24 hours after the virus. Hemagglutinin titrations were made in the usual way about 44 hours after the injection of virus. It was found that marked inhibition occurred when the compound was injected an hour ahead of the virus. When the compound followed the virus by 2 hours significant inhibition was still observed, but when the interval was increased to 24 hours, the effect was variable and of doubtful significance. Such results could conceivably have been due to interference by canavanine with adsorption of the virus to the host cells, or possibly to some direct inactivating effect of the compound on the virus. Both of these possibilities were investigated.

To determine any possible effect upon adsorption of the virus, fresh allantoic fluid virus was diluted 2 fold and 5 fold with buffered saline and then incubated at 35°C for 30 minutes in the presence of fresh, washed chorioallantoic membrane tissue in the proportion of 1 g per ml of diluted virus. One set of such preparations contained canavanine at a concentration of 4 mg per ml, while a parallel set contained none of the compound. Controls containing only the virus, and containing the virus plus canavanine without tissue were included. The pH of the preparations containing canavanine was very slightly more alkaline than those without it (*e.g.* 7.7 as compared to 7.5). Hemagglutinin titrations were made before and after the 30 minute incubation period. It was found that with the 1:2 dilution of the virus about 87% of the virus was adsorbed by the tissue, and identical results were obtained whether canavanine was present or not. With the 1:5 virus dilution about 95% of the hemagglutinin was adsorbed in both cases. Thus the evidence indicated that under these experimental conditions at least, canavanine did not cause any significant interference with adsorption of the virus by the tissue.

To reveal any possible *in vitro* inactivating effect of canavanine on the Lee influenza virus, a sterile concentrated solution of the compound was added to fresh allantoic fluid virus to give a concentration of 4.0 mg per ml. This was the approximate concentration estimated to result in the allantoic fluid of chick embryos when a dose of 20 mg of the compound was injected. The virus containing canavanine was divided into two parts, one of which was incubated at 10°C for 24 hours, while the other was held at 35°C for the same period. Parallel preparations containing no canavanine were similarly treated. The pH of the virus alone before incubation was 8.3, and that of the virus plus canavanine was 8.2. All preparations were tested for bacterial sterility before and after incubation and found to be free from viable bacteria. Both hemagglutinin and infectivity titrations were carried out before and after the incubation period to determine whether the compound had any effect on either of these viral properties. The results of a typical experiment are presented in Table II.

It is evident that canavanine at this concentration produced no effect on the hemagglutinating activity of the virus within 24 hours at either temperature. With respect to infectivity, the preparation containing canavanine and held at 10°C showed a slightly lower activity than the control preparation, but the difference is not great enough to be significant. Furthermore, it has not appeared in either of 2 other experiments carried out in the same way. In the case of the preparations held at 35°C, the control containing no canavanine decreased in infectivity by about

TABLE II. *In Vitro* Effect of Canavanine on the Lee Influenza Virus.

Preparation	Time and temp.	pH	Hemagglutinin titer	EID_{50} of virus/ml
Afv[*]	Before incubation	8.32[†]	640	$10^{9.3}$
Afv	24 hr, 10°C	8.67	480	$10^{9.4}$
Afv + C, 4 mg/ml	*Idem*	8.52	"	$10^{8.70}$
Afv	24 hr, 35°C	8.86	640	$10^{2.48}$
Afv + C, 4 mg/ml	*Idem*	8.54	"	$10^{5.5}$

[*] Afv = allantoic fluid virus; C = canavanine.

[†] The pH of virus + canavanine before incubation was 8.22. All preparations were free from viable bacteria.

7 log units during the 24 hours, while the virus containing the canavanine dropped only about 4 log units in infectivity. Thus, the compound appeared to exert a stabilizing rather than an inactivating effect. The same effect was observed in each of 3 experiments. It did not appear to be due entirely to the buffering effect of the canavanine. In any case the experiments indicated that canavanine in a concentration found to be inhibitory to the growth of the virus in the chick embryo, did not exert any inactivating effect on the virus within 24 hours at 35°C *in vitro*. Thus the inhibition of the multiplication of the virus in the chick embryo cannot be explained on this basis.

It appeared theoretically possible that the effect of canavanine upon the multiplication of the Lee virus in the chick embryo might be due to interference with release of the virus from infected host cells. If such were the case, then in canavanine treated, virus infected embryos, the virus content of the cells of the chorioallantoic membrane might be expected to be comparable to that of untreated infected embryos, even though the virus content of the allantoic fluid was much lower in the treated group. This point was examined by measuring the virus concentration of membranes from infected embryos treated with 20 mg of canavanine an hour before the injection of 50 EID_{50} of virus, and from a similar group of untreated infected embryos. After 44 hours incubation, fluids and chorioallantoic membranes from all eggs were harvested. A 10% suspension of each membrane was prepared in physiological saline in a Waring blendor and hemagglutinin titrations were run on each suspension, as well as on each specimen of fluid.

The virus content of the pooled fluids from canavanine-treated eggs was lower than that of the controls by a factor of >250. The tissue suspensions from the control group when pooled showed a hemagglutinin titer of 60, while a similar preparation from the treated group showed no activity when tested without further dilution. Thus it appeared that the virus content of the membranes of canavanine-treated eggs was also markedly lower than that of control eggs, and there was no evidence that canavanine acts by interfering with release of virus from the host cells. To gain further insight into the mechanism of the inhibition, the effect of canavanine on the multiplication of the Lee virus in tissue culture was studied. The technic employed portions of chorioallantoic membrane tissue suspended in a buffered glucosol and incubated at 35°C for 44 to 48 hours on a shaker, as previously described. An inoculum of 0.1 ml of a 1:10 dilution of fresh allantoic fluid virus was added to each culture containing 2.0 ml of buffered glucosol. The virus content of each culture after incubation was determined by hemagglutinin titration of the culture fluid. A 0.5% suspension of washed chicken erythrocytes was employed in this case, instead of the 1.0%, in order to increase the sensitivity of the test.

Inhibition of the virus by canavanine in tissue culture was demonstrable at considerably lower concentrations of the compound than in the case of the intact chick embryo. In the latter case, complete suppression of hemagglutinin development was not always achieved even with a dose of 20 mg per egg, which probably produced a concentration in the allantoic fluid of about 3 to 4 mg per ml. In tissue culture, however, more complete suppression of hemagglutinin occurred in the presence of 0.2 mg of canavanine per ml. Slight inhibition was noted with concentrations as low as 0.02 mg per ml. The inhibitory effect obtained at 3 different concentrations of canavanine is shown in Table III. In view

TABLE III. Reversal of Canavanine Inhibition of Lee Influenza Virus in Tissue Culture by Arginine.*

		Hemagglutinin titers in cultures containing						
				Arginine : canavanine ratio in mg/ml				
Exp. No.	Conc. of canavanine, mg/ml	No canavanine; no arginine	Canavanine only	1:1	3.5:1	4.6:1	7:1	14:1
LC-32	.1	160	80	240	160		240	
		160	60	160	80		160	
		120	40	160	80		160	
		80	40	160	80		160	
		60	5	160	40		160	
		40	2	80	40		80	
	Geom. mean	88.1	20.6	152.5	71.3		152.5	
-24	.2	120	4	80	120		120	
		120	4	20	60		60	
		80	2	20	40		40	
		80	2	20	40		30	
		80	0	2	30		30	
		60	0	0	2		20	
	Geom. mean	87.3	2	10.4	29.7		41.6	
-36	.3	160	5			160	240	60
		160	0			160	160	60
		120	0			120	80	60
		120	0			80	60	40
		80	0			60	30	30
		80	0			40	5	5
	Geom. mean	115.4	1.3			91.6	55.0	33.0

* L-arginine monohydrochloride.

All tissue cultures inoculated with 0.1 ml of a 1:10 dilution of fresh allantoic fluid virus. In any experiment virus inoculum was constant. Some variation existed between different experiments. However, initial hemagglutinin titer after inoculation and before incubation was not greater than 2. Based on usual infectivity titers obtained with fresh virus ($10^{8.5}$ EID_{50}/ml) inoculum present in tissue cultures initially could be estimated at about $10^{6.2}$ EID_{50}/ml.

of the fact that canavanine has been shown to be a competitive antagonist of arginine for certain microorganisms(1,2) it was desirable to determine whether a similar mechanism might be responsible for the inhibition of the Lee influenza virus by this amino acid. Accordingly, tissue cultures were set up containing canavanine in various concentrations found to be inhibitory to the virus, and also L-arginine monohydrochloride in various ratios to the canavanine. The compounds were dissolved in the buffered glucosol at the desired concentrations and the solution sterilized by filtration through a sintered glass filter before dispensing in the culture tubes. The results of 3 representative experiments are presented in Table III.

At a canavanine concentration of 0.1 mg/ml, partial inhibition of hemagglutinin development occurred and that this was completely reversed by arginine. In fact, at the 1:1 and 7:1 ratios of arginine to canavanine the virus titers appeared to be higher than in control cultures, but these differences were not found to be statistically significant. At this low concentration of canavanine, the ratio of arginine to the inhibitor required for complete reversal of the inhibition did not appear to be critical. With canavanine at 0.2 mg per ml, inhibition was marked and the data indicate that an arginine canavanine ratio of 7 to 1 produced more complete reversal than lower ratios. In other experiments greater amounts of arginine were not more effective than the 7 to 1 ratio at this canavanine concentration. When the latter was increased to 0.3 mg /ml marked inhibition of the virus was again observed, and essentially complete reversal was noted when arginine was present in a concentration 4.6 fold greater than the canavanine concentration. Increasing the ratio up to 14 to 1 resulted in less complete reversal of the inhibition. Some inhibitory effect from the arginine itself was probably beginning to occur at this point(3). These results indicate that the mechanism by which canavanine inhibits the development of the Lee influenza virus in tissue culture is antagonized by L-arginine. The data are not adequate to determine whether this is a truly competitive type of inhibition. However, the ratios of arginine to canavanine found to be adequate for reversal of the inhibition are similar to those observed in work with canavanine and certain microorganisms(1,2). Similar experiments carried out with L-lysine monohydrochloride and L glutamic acid hydrochloride failed to show any reversal of the inhibitory effect of canavanine.

Experiments were performed in the conventional Warburg respirometer to determine whether canavanine exerted any influence on the respiration of chorioallantoic tissue. Some experiments were conducted in the usual manner in the absence of CO_2; in other experiments the technic of Pardee(10) was employed to provide an atmosphere of approximately 3% CO_2 in the respirometer flasks. Canavanine concentrations of 0.1 and 0.2 mg per ml were employed. Respiration was observed during the first 8 hours and in some cases for similar periods after the flasks were 24 and 48 hours old. However, the respiratory rates observed in the presence of the compound did not differ significantly from the control flasks containing only the tissue. Apparently the mechanism of inhibition of the virus does not depend upon depression of the respiration of the host cells.

In view of the inhibitory activity of canavanine for the Lee influenza virus it was of interest to determine whether the compound could influence the multiplication of other viruses. Accordingly, it was tested against the PR8 strain of influenza A, and against the mumps virus, using the intact chick embryo technic as previously described. However, no significant inhibitory effect could be noted against either of these agents. This observation offers further evidence of the rather specific nature of the inhibition of the Lee virus by canavanine.

It also appeared desirable to determine whether this compound could influence the course of experimental infection of the mouse with the Lee virus. Accordingly, 5 groups of 10 Swiss mice ranging in weight from 16 to 19 g were selected. Two of the groups were inoculated intranasally under light ether anaesthesia with approximately 30 mouse ID_{50} of Lee virus in the form of mouse lung suspension. The mice in one of these groups received an intramuscular injection of 15 mg of canavanine in a repository form immediately after inoculation of virus, and this injection was repeated daily for 10 days. Two other groups of mice were inoculated with approximately 3 mouse ID_{50} of virus. One of these groups received the daily treatment with canavanine. The fifth group received no virus but only the daily injection of canavanine. The canavanine was prepared as a suspension in sterile sesame oil containing 2% of aluminum monostearate. The concentration of canavanine was 300 mg per ml. The daily dose given to the mice was about the maximum that was compatible with the physical limitations of the preparation and route of injection, and represented over 800 mg per kg per day. At the end of the 10-day period of treatment and observation no significant differences could be noted between the infected groups of mice regardless of whether they had been treatd with canavanine or not. Most of them died before this time and among the few remaining alive the extent of lung lesions was comparable in both treated and untreated mice. Nine out of 10 animals in the control group survived the daily injections of canavanine.

Discussion. It appears of some theoretical interest that the multiplication of the Lee influenza virus is inhibited by the amino acid, canavanine, and that this inhibition is specifically reversible by arginine. In view of the fact that *Neurospora,* certain lactobacilli and *Escherichia coli* are similarly inhibited and that these inhibitions are also reversible by arginine(2,3), the evidence suggests that there may be an important reaction which is critical for the growth of these microorganisms which is also involved in the formation of the Lee virus. If this is true, however, such a reaction does not appear to be critical for the formation of the PR8 or mumps viruses.

The work reported here shows that canavanine does not exert any inactivating effect on the Lee virus *in vitro*, and has no demonstrable effect on the adsorption of the virus to the host cells. It does not appear to interfere with the release of virus from infected host cells. It does not depress the respiratory rate of chorioallantoic tissue in concentrations which are inhibitory to the virus. It is most effectively inhibitory to the virus in the chick embryo when administered an hour before the virus, but it need not be injected by the same route. In tissue culture the inhibition of the virus can be completely reversed by the amino acid arginine, but not by lysine or glutamic acid.

All of these observations seem to be most readily compatible with the hypothesis that canavanine interferes in some manner with the formation of new virus. The specific reversing effect of arginine suggests that a reaction involving the latter amino acid is the basis for the inhibition. The data at hand suggest that such a reaction probably involves the utilization of arginine, rather than its synthesis.

Summary. The amino acid, L-canavanine, has been found to inhibit the multiplication of the Lee influenza virus in the chick embryo and even more effectively in tissue culture. It had no direct inactivating effect on the virus, and did not interfere demonstrably with the adsorption of the virus to the host cells. It did not interfere with respiration of the host cells. In tissue culture the inhibitory effect of the compound on the growth of the virus was completely reversed by L-arginine. The implications of these observations with respect to the possible mechanism of inhibition are discussed.

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Demonstration of Two Effects of Cortisone on Virus-Cell Interaction.

(21499)

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It has been shown that cortisone enhances viral and rickettsial infections in animals (1-7). The possible enhancement of tissue culture infection seemed worthy of investigation because such effect could facilitate isolation of viruses and rickettsiae, and simplify studies of the mechanism of the hormone action.

For these reasons a study of the effect of cortisone on interaction of several viruses and HeLa cells was initiated. To our knowledge, the only publication describing the use of this substance in the *in vitro* growth of viruses has been Kilbourne and Tateno's report that cortisone enhances the multiplication of influenza B virus in fragments of chorioallantoic membrane suspended in a mixture of equal volumes of modified glucosol solution and phosphate buffer(8). The experiments to be described have revealed that, depending on the time that the hormone is introduced into the culture, cortisone may exert opposite effects on the development of virus-induced cytopathogenic changes.

Materials and methods. Viruses. Poliomyelitis virus Type I (Mahoney strain)* of 4th and 5th HeLa passages derived from 17th local passage in monkey testes tissue culture. Poliomyelitis virus Type II (YSK strain)* of 5th HeLa cell passage subsequent to 11 passages in monkey testes culture. Vaccinia virus, CDC Lab. No. 8014 of 2nd HeLa passage following one passage in rabbit testicle after isolation on chorioallantoic membrane. *HeLa cells* were derived from flask cultures kindly supplied by Dr. Russell Brown of the Carver Foundation, Tuskegee Institute. The method of growing and maintaining these cultures was essentially that described by Syverton, Scherer, and Elwood(9), with two exceptions. Ten per cent rabbit serum was substituted for chicken serum in maintenance medium and the concentration of glucose was reduced to 0.1%. In our laboratory rabbit serum medium appeared to be superior to chicken serum as regards appearance and survival of cultures, though with the rabbit serum medium there was some multiplication of cells. *Cortisone* (11-dehydro-17-hydroxycorticosterone-21-acetate, Merck) lot numbers OIC 2555, 11C 0008,† and OIC 3064 were used and gave essentially the same results. *Vehicle* No. 1† (Merck), used in commercial preparation of cortisone suspensions, served as an added control in several experiments.

Results. Effect of cortisone on virus-cell interactions during infection. In a preliminary experiment, cortisone in final concentration of 0.05, 0.1, 0.25, and 0.5 mg/ml was added to cultures immediately prior to infection with approximately 500 cytopathogenic (CP) doses of poliomyelitis virus Type I (based on 50% endpoint). The two lowest concentrations had little or no effect on the progress of infection. The highest concentration was toxic for cells. By the second day, cultures containing 0.5 mg of cortisone showed a predominance of abnormal cells which varied in appearance from "rounded" to "stringy"; the following day the cells were extremely granular and tended to clump. There was a marked decrease in pH and a considerable loss of cells. In the presence of 0.25 mg cortisone the cultures were quite normal in appearance except for increased granularity, some of which seemed to represent extracellular particles of cortisone, and for a more rapid decrease in pH of the maintenance medium than in untreated controls. This concentration of cortisone caused a delay in development of viral CP changes. In most subsequent experiments the cortisone dosage used was 0.25 or 0.3 mg/ml. In every one of 9 experiments employing several dilutions of poliomyelitis virus Type I the hormone delayed or inhibited the CP changes. This effect was manifested by (1) a larger proportion of cultures showing little or no microscopic evidence of viral activity at early intervals, (2) an increase in time required for complete destruction of the cultures, and (3) a smaller proportion of cultures with significant degree of degeneration (3-4+) as compared with untreated cultures. Vehicle alone was without effect. The same inhibitory action of corti-

TABLE I. Effect of Cortisone in Maintenance Solution on Cytopathogenic (CP) Changes Produced by Poliomyelitis and Vaccinia Viruses.

Test virus*	Virus dilution	No. of cultures with significant CP changes†		Avg time in days to reach significant CP change	
		Untreated	Cortisone	Untreated	Cortisone
P, type I	10^{-2}	8/8	8/8	2.6	3.5
	10^{-3}	8/8	6/6	3.0	4.2
	10^{-4}	8/8	4/6	3.2	5.0
	10^{-5}	7/8	0/5	4.3	>5.0
P, type II	10^{-2}	5/5	3/3	3.0	4.0
V	10^{-1}	4/4	4/4	4.2	5.2
	10^{-3}	4/4	3/4	6.2	8.0

* P = poliomyelitis; V = vaccinia.

† Significant refers to 75-100% destruction of cells; period of observation for poliomyelitis Types I and II, 5 days; for vaccinia, 8 days.

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* Originally obtained from Dr. Jonas Salk.

† Received through the courtesy of Merck and Co., Rahway, N. J. The vehicle consists of saline solution containing "suspending agents" and 0.9% benzyl alcohol.

TABLE II. Lack of Direct Action of Cortisone on Poliomyelitis Virus Type I.

		Exposure time prior to inoculation					
		0 hr		24 hr		48 hr	
Virus dilution	Treatment of virus*	Proportion of 3-4+ cultures	Avg time in days to reach 3-4+ CP change	Proportion of 3-4+ cultures	Avg time in days to reach 3-4+ CP change	Proportion of 3-4+ cultures	Avg time in days to reach 3-4+ CP change
10^{-4}	No C	3/3	3.0	3/3	3.0	3/3	3.3
	C uncentrifuged	3/3	5.7	3/3	6.0	3/3	5.0
	C†	3/3	3.0	3/3	3.0	3/3	3.0
10^{-5}	No C	3/3	4.0	3/3	3.7	2/3	3.5
	C uncentrifuged	2/3	6.0	3/3	6.0	1/3	5.0
	C†	3/3	3.0	3/3	3.3	3/3	4.3

* C = Cortisone. † Centrifuged before inoculation to remove cortisone suspension.

sone was observed in experiments employing poliomyelitis virus Type II and vaccinia virus. Table I illustrates the results of typical experiments with each of these viruses.

Lack of direct action of cortisone on virus. To determine whether the action of cortisone could be attributed to possible adsorption or destruction of virus, the following experiment was designed. Serial 10-fold dilutions of poliomyelitis virus Type I were prepared in maintenance medium. Each dilution was divided into 6 aliquots, half of which received 2.5 mg of cortisone per ml and the other half a corresponding volume of maintenance solution. One set of aliquots (one with cortisone, one without) was inoculated into tissue cultures immediately. The second set was kept at 36°C for 24 hours and the third set incubated 48 hours. Immediately prior to inoculation, tubes containing cortisone were further subdivided into 2 portions, one centrifuged at 1500 RPM for 15 minutes to remove cortisone particles. Thus, each dilution of virus was represented in 3 preparations at each of the 3 time intervals. The findings are given in Table II. From the results obtained with the centrifuged preparations it appears that the virus was neither adsorbed nor destroyed by cortisone even after contact for 48 hours. Preparations in which cortisone was not removed by centrifugation prior to inoculation caused typical delay in CP activity. In view of the toxic effect of higher concentrations of cortisone, it was deemed desirable to determine whether cells which had been maintained in the presence of cortisone for several days would be capable of further multiplication. For this reason, subcultures were made from cells exposed to 0.25 mg of cortisone for several days. These yielded satisfactory cultures. Attempts to determine whether the inhibitory effect of cortisone was still present in these subcultures yielded the surprising finding that on challenge these cultures appeared to be more susceptible to the CP action of virus than were subcultures from untreated cells. As a result of these preliminary observations several experiments were performed in which cortisone was incorporated into the growth medium at initiation of new tube cultures.

Effect of incorporation of cortisone in growth medium on subsequent virus-cell interaction. A pooled suspension of HeLa cells was obtained from flask cultures. After proper dilution of the suspension to give 100,000 cells/ml, it was divided into 3 portions which received (a) 0.25 mg/ml cortisone, (b) an equivalent volume of vehicle, and (c) an equivalent volume of maintenance solution, respectively. Each preparation was dispensed in 0.5 ml amounts into screw cap tubes. Following growth for 4 to 5 days, the cultures were washed 4 times with 1 ml of balanced salt solution and to each tube was added 1 ml maintenance solution. Inoculations with virus were usually carried out on following day.

The results obtained with poliomyelitis virus Types I and II are given in Table III. In every instance there was a pronounced acceleration of the CP process in cultures which had been grown in the presence of cortisone. Results with cultures grown in the presence of

TABLE III. Effect of Incorporation of Cortisone in Growth Medium on Subsequent Infection of HeLa Cells with Poliomyelitis Virus Types I and II.

Test virus*	Virus dilution	Avg time in days to reach significant CP change Cells grown without cortisone	Cells grown with cortisone
P, type I	10^{-3}	3.2	2.4
	10^{-4}	4.6	3.0
" II	10^{-3}	4.7	3.0
	10^{-4}	5.3	4.3

* P = Poliomyelitis.

cortisone vehicle were comparable to those of normal controls. The results obtained with vaccinia virus have been irregular and require further investigation.

Discussion. The results of this study revealed opposite effects of cortisone on virus-cell interaction. Cortisone appears to alter the metabolic activity of cells, as evidenced by an accelerated decrease in pH and by the development of abnormal morphologic changes with higher concentrations of hormone. The nature of the factors involved remains to be explored. It will be of especial interest to determine whether the opposite effects are due to the hormone's influence on the same or different factors during cell multiplication and during infection.

So far we investigated the possible reversibility of "increased susceptibility effect" in cells grown in presence of cortisone and then removed from cortisone. Based on two preliminary experiments it would appear that this effect could be overcome by addition of cortisone to cells at the time of infection.

Summary. Cortisone in a concentration of 0.25 mg/ml produced a delay and partial inhibition of CP changes when introduced into HeLa cell cultures at the time of infection with poliomyelitis virus Types I and II and with vaccinia virus. This substance appears to have no effect on the virus in the absence of living cells. When cortisone in the same concentration was added to actively growing HeLa cells and then washed away prior to infection, it accelerated the CP changes when cells were subsequently infected with poliomyelitis virus Types I and II.

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Local Inflammatory Response Produced by Solutions Sterilized with Asbestos Type Filters.* (21500)

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The peritoneal cavity has been demonstrated to be an excellent site for studying the local cellular reactions to various drugs, hormones and other physiologically active substances. Samples of peritoneal fluid can be easily taken and cell counts performed by using slightly modified hematological technics. In this manner, quantitative as well as

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TABLE I. Local Cellular Effect of Intraperitoneal Injections of Heparin and Saline Solutions. (Cell counts taken of peritoneal fluid 24 hr after injection.)

Material inj.	Dose	No. of mice	Cellular response		
			Total cells/mm^3	Eosinophils/mm^3	% eosinophils
Non-inj. mice	—	12	128,605 ± 11,136	3,729	2.9
Heparin in .2 ml saline*					
a. Not sterilized	.2 mg/.2 ml	8	150,000 ± 20,219	2,581 ± 486	1.7
b. Sterilized with fritted glass filter	"	3	178,583	2,983	1.7
c. Sterilized with Seitz filter (E.K.)	"	9	175,629	64,983 ±17,130	37.0
Saline*					
a. Unfiltered	.2 ml	9	129,167 ± 17,469	1,844 ± 250	1.5
b. Seitz filtered	"	8	164,238 ± 14,553	18,597 ± 3,318	12.9
c. Filter fibers added (approx. 1 mg/ml)	"	9	163,700 ± 23,212	46,495 ± 4,083	32.3

* 0.85% sodium chloride, pyrogen free (Abbott).

qualitative changes of the cells found in the peritoneal fluid can be determined following an intraperitoneal injection(1). In an attempt to characterize the cellular responses to heparin and other substances, the authors noted that certain solutions produced local reactions which were markedly different from the controls. These solutions had been sterilized by filtration thru a Seitz E.K. asbestos filter disc. It was found that the use of this type of filter disc resulted in solutions contaminated with filter particles which produced very misleading results and necessitated months of additional work. It is possible that many other laboratories and clinics are experiencing some of the same difficulties. For example Bilimoria reported(2) that he obtained a histamine like "flare" to intracutaneous injections of a vaccine which had been Seitz filtered. The unfiltered vaccine did not produce this response, nor was histamine detectable in the filtrate using the diazo-test.

This paper reports some of the local reactions produced by solutions which had been passed thru asbestos-paper type filter discs (Seitz E.K.).

Materials and methods. The animals used in these experiments were normal BBF_1 male mice between 2½ and 4 months of age, housed 3 mice per cage in the animal room and fed laboratory chow *ad lib*. Each animal was used once and then discarded. Injections were made into the ventral aspect of the peritoneal cavity with sterile needle and syringe. Peritoneal fluid samples were taken under ether anesthesia with an RBC pipette through a ventral midline incision. The fluid was diluted 1 to 200. The total cell counts were made with a 35% acetone toluidine blue diluent(3), and immediately loaded and counted in an 0.2 ml counting chamber(6). Eosinophil counts were made in the same manner using a 15% acetone-phloxine diluent(4,5). The *heparin* used in these experiments was furnished by Hynson, Westcott, and Dunning Pharmaceutical Laboratory, but heparin from other sources gave similar responses. It was dissolved in a pyrogen free solution of sodium chloride for intravenous injection (Abbott & Co.). The pollen used was a mixed short and giant ragweed pollen extract, 1:100 of 10,000 units/ml (Abbott & Co.). This was diluted 1 to 30 with sterile saline before using. The asbestos and talc were obtained from several commercially available preparations and all produced very similar responses. Suspensions of asbestos and various other minerals were sterilized by autoclaving before using. The remaining solutions were sterilized by filtration under a partial vacuum, using a Seitz-Uhlenhut-Manteufel Filter with a 6 cm Seitz E.K. asbestos filter disc. In the case of heparin a pyrex glass filter with a fritted disc was also used.

Results. Table I shows the data obtained when cell counts were made of the peritoneal fluid of uninjected mice and mice which had received an intraperitoneal injection of he-

TABLE II. Nature of Chemotactic Substance in Asbestos Filter Discs. (Cell counts taken 24 hr following a single I.P. injection.)

Material inj.	Dose*/inj. (0.2 ml)	No. of mice	Cellular response		
			Total cells/mm^3	Eosinophils/mm^3	% eosinophils
Suspension of filter fibers after boiling 20 min.	.2 ml	3	123,968	38,792	34.0
Filtrate after paper filtering	.2	3	99,711	4,550	4.7
Suspension of ashed filter fibers	.4 mg	6	202,916 ± 47,655	45,890 ± 2,813	25.0
Water insoluble fraction after washing and resuspending	.4	3	230,750	49,420	16.0
Soluble fraction	.2 ml	3	115,666	2,083	1.7
Ashed filter fibers ground and suspended	.01 mg	6	116,375 ± 10,363	3,100 ± 591	2.8
	.1	9	242,777 ± 30,921	45,658 ± 9,235	18.6
	.4	6	179,776 ± 33,823	40,371 ± 12,478	22.3
	.8	7	410,526 ± 87,829	60,992 ± 7,310	17.3
	6.4	9	92,839 ± 11,975	7,256 ± 1,699	7.6
American asbestos (Fisher) Acid washed	.4	6	290,074 ± 38,717	71,792 ± 6,635	24.8
Silica, 140 mesh (Fisher)	.4	3	100,338	2,900	2.9
240 ”	.4	3	132,832	7,517	5.7
Cotton fibers, absorbent (J & J)	.4	6	158,047 ± 15,460	2,183 ± 420	1.6
Paper fibers (shredded filter pad)	.4	6	170,583 ± 14,516	3,950 ± 900	2.5
Glass wool (commercial)	.4	9	128,851 ± 11,458	13,767 ± 5,288	9.7
Mica ”	.4	6	138,083 ± 14,900	11,662 ± 3,140	8.7
Talc (French chalk) commercial	.4	13	253,884 ± 29,923	66,253 ± 11,211	26.5

* Vehicle was 0.85% sodium chloride, pyrogen free (Abbott).

parin or saline. It may be seen that 24 hours after the injection there was an increase which was not significant in the total number of cells per cubic millimeter. The eosinophils however, were significantly changed following injections of solutions which had been Seitz filtered. In other experiments it was observed that this local accumulation of eosinophils began within 4 hours after the injection of heparin and persisted for at least 7 days. A slight neutrophilia also occurred which disappeared within 48 hours. The local increase in eosinophils caused a great deal of concern until it was discovered that the effect was due to the manner in which the heparin or saline had been sterilized and was not a normal response of the animal to these solutions. The response could be produced merely by adding a small quantity of filter fibers to the solution before injection.

Additional experiments were set up to determine the factors involved in producing this eosinophilic response. The results of these experiments are shown in Table II. It can be seen that if the filter fibers are boiled for 20 minutes in saline and the suspension of fibers injected into the peritoneal cavity, a marked local eosinophilia occurs. However, if the fibers are removed and only the filtrate injected, the eosinophilia does not occur. This indicates that the eosinophil-tactic material is not soluble in water. When the filter pads were ashed over a bunsen burner, the activity was found in the remaining insoluble material which is asbestos. In addition it was observed that the eosinophilia occurred only in specific concentrations of the ashed filter fibers. Injections of from 0.1 mg to 0.8 mg resulted in a local eosinophilia. Low doses such as 0.01 mg and very high doses such as 6.4 mg produced little or no eosinophilia. Injections of asbestos produced essentially the same response as did the filter fibers. However, injections of cotton and paper fibers, silica and

TABLE III. Local Effect of a Single Previous Injection of Filter Fibers upon Cellular Response to Multiple Injections of Pollen. (Cell counts taken 24 hr following last I.P. inj.)

			Cellular response		
Material inj.	Ashed filter fibers added	No. of mice	Total cells/mm^3	Eosinophils/mm^3	% eosinophils
.2 ml pollen extr. 1:100 (Abbott)	0	16	238,844 ± 21,603	21,370 ± 3,206	9.7
Dil. 1/30 with saline*	.4 mg/.2 ml	16	273,312 ± 28,777	52,093 ± 6,330	20.3

* 0.85% sodium chloride, pyrogen free (Abbott).

many other irritants in various concentrations had no marked eosinophilic effect. Glass wool fibers and mica produced a slight eosinophilic response. Talc on the other hand, produced a very high local eosinophilia, similar to the asbestos. Asbestos and talc are very much alike in their chemical and biological activity. They are both soft fibrous minerals made up primarily of magnesium silicate along with variable amounts of calcium, potassium, iron, and other metal compounds. Both substances also have been reported to produce nodular formation at the site of injection(9) and pneumonokoniosis after inhalation(10,11). Thus a local accumulation of eosinophils at the site of injection is a specific reaction to a substance common to talc and asbestos and is not a non-specific response to irritation.

In the final experiment, an attempt was made to determine whether the filter fibers would change the local response to antigenic material such as ragweed pollen extract. The filter fibers were ashed and then single injections of 0.4 mg were made into the peritoneal cavity of normal mice. The animals were then given a series of sensitizing injections of pollen (6 to 11) over an 8-week period. At various intervals peritoneal counts were taken and compared with a similar group of animals receiving only the pollen injections. The data are shown in Table III. It may be seen that repeated injections of pollen produced a local eosinophilia as reported earlier(1). However, the addition of a single injection of filter fibers, significantly increased the number of local eosinophils. It is interesting to note that this eosinophilic effect was observed in other experiments for as long as 14 weeks after the injection of the filter fibers.

Discussion. Experiments reported in this paper have clearly demonstrated that solutions sterilized through asbestos type filter pads will produce local cellular responses different from those produced by the same solutions sterilized by other methods or unsterilized. Heparin and saline solutions filtered through a Seitz filter disc contained asbestos particles which produced a tremendous increase in the number of eosinophils at the injection site.

It is known that injections of foreign material will produce an inflammatory response which is characterized by a local neutrophil leucocytosis followed by a local accumulation of other cells. These cells form the first line of reaction of the organism to an injected material, and the effect of that material on the organism as a whole, depends to a great extent upon the rapidity and type of local reaction. It would be logical to expect that the change in the local reaction to an injection would produce a change in the effect of the injected solution on the organism as a whole. This has been clearly shown in immunological experiments in which adjuvants have been added to antigens, to intensify and prolong antibody production(7,8). It was noted in this paper that the addition of asbestos fibers produced a significant increase in the number of local eosinophils following injections of pollen antigen. It is not known at this time what effect this local eosinophilia has on the reaction of the host to the antigen or vice versa. However, it is possible that the local effect of asbestos fibers could change the effect of injected material on the organism and so could be very misleading to the allergist who is using skin tests to determine sensitivity of a patient. This is brought out by the fact that a Seitz filtered solution can produce a histamine-like "flare" to intracutaneous injections, even though histamine is not detectable(2).

The harmful action of talc in the peritoneal cavity has been known for years. Seelig, Verda and Kidd(12) pointed out that the use of talc was a "grave menace to surgery."

Asbestos also must be considered as a potential danger and precautions must be taken to avoid accidental introduction of these materials into serums and other biological materials for injection. At the present time, the manufacturers have indicated that it is not feasible to incorporate a paper filter into the asbestos pad as one integrated unit. However, it may be possible to place a filter paper beneath the asbestos filter pads and thus prevent the asbestos fibers from passing into the filtrate.

These experiments have demonstrated how a study of the local cellular responses to an injected material can lead to better understanding of the effects of a solution upon an organism. It would seem desirable that all materials for injection should be studied in this manner to determine their local effects. This applies particularly to materials recommended for intravenous injection, allergans, etc.

Summary: 1. This paper demonstrates that filtering through an asbestos type filter disc will change the local cellular responses of the organism to that solution. This reaction is due to particles and fibers which have pulled off the disc and are present in the filtrate at the time of injection. 2. An attempt was made to characterize this reaction and resolve the factors involved in its production. The reaction was manifested by a tremendous increase in number and percent of eosinophils at the injection site. The particles producing this reaction were found to be insoluble in water and not destroyed by ashing. Identical reactions were obtained when asbestos fibers from 3 different sources were substituted for the filter fibers. Thus the local eosinophilia was due to the presence of asbestos fibers in the injected filtrate. Further studies indicated that the eosinophilia was not merely the reaction to a non-specific irritating substance. Talc produced a response identical to the asbestos, glass fibers and mica produced only a slight response and other substances such as silica, cotton and paper fibers did not produce an eosinophilia. Further studies are being made in order to determine the specific chemical producing the eosinophilia. 3. Asbestos fibers must stand with talc as potentially harmful irritants when introduced within a patient or animal. Immunologists and allergists as well as other biologists and physicians using asbestos type filters should take precautions to avoid introducing asbestos fibers along with their injections. It was further noted that a study of the local cellular responses to intraperitoneal injections, can lead to a better understanding of the effects of a solution upon the organism.

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Increased Anoxic Resistance Resulting from Short Period Heat Adaptation. (21501)

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The writers use the term adaptation to indicate short term periods of physiological adjustment as opposed to acclimatization for long periods involving evolutionary changes as suggested by Monge(1) and Sealander(2). Adaptation to cold which can take place in a few hours has been shown to increase tolerance to hypoxia(3). Also lowered ambient temperature increases hypoxic tolerance which has been measured by survival at simulated altitude(4-7), by reduced oxygen consumption(8-9) or by drowning(10-14). Adaptation to heat is less well understood because most investigators have been primarily interested in the effects of cold. However, as heat is a form of stress some sort of adaptation should result from heat exposure. Two possibilities suggest themselves, 1) a decreased anoxic resistance due to excitation caused by the stress increasing adrenal activity or, 2) an increased anoxic resistance due to the increased temperature by way of depression of the thyroid gland, commonly termed "involution of the thyroid." That depression of thyroid activity increases hypoxic resistance has been demonstrated by thyroidectomy and by the anti-thyroid drugs(15-19). Morphological changes in the thyroid and adrenals have been seen with heat adaptation (20) as well as decrease in weight of testes and seminal vesicles(21).

Methods. Two groups of 12 male Purdue Swiss white mice of approximately similar weight were placed in a constant temperature incubator with food and water at their disposal and kept at a temperature of 36-37°C for 2 periods of time. Group A was kept for 10 days and group B for 14 days. At the end of each time period the mice were removed and individually drowned by immersion in a water bath of 37.5°C which temperature being close to the body temperature of the mouse does not introduce a separate factor of a different ambient temperature(14). Death was taken arbitrarily as the last visible gasp or any other movement which was timed to the nearest second. Drowning was accomplished by seizing the tip of the mouse tail with a long hemostat and plunging the animal under water. With a little practice one becomes proficient in judging the time of the last movement. Two observers watched each drowning procedure and usually agreed upon the time within one second. Control mice which had been kept at room temperature (24-25°C) for the same length of time as the experimentals were drowned in exactly the same way as the experimentals. External factors were maintained identically with experimental and control animals with the exception of the ambient temperature.

TABLE I. Drowning Time of Male Purdue Swiss Mice in Water of 37.5°C after Adaptation to Incubator Temperature of 36-37°C. All figures are averages.

	No. of animals	Drowning time, sec.	Variation from controls, %
Controls	31	42.1 ± 3.5*	
Group A	12	48.2 ± 5.9	+14.5
" B	12	54.3 ± 4.2	+28.9

Group A were acclimatized for 10 days, Group B for 14 days.

$$* \sigma = \sqrt{\frac{\Sigma(\Delta^2)}{N}}.$$

Results: The results of heat adaptation on drowning survival are shown by Table I. The 14 day heat-exposed mice were about twice as effectively adapted as were the 10 day exposed animals as judged by the percentage increase in survival time. Other adaptive changes were shown by the pendulous position of the scrotum, piloreotion of the pelage and erythematous condition of the ears and tail. The mice of group B lost weight progressively during the exposure to heat. At the beginning of the experiment the total weight of the 12 mice was 368.0 g, at the end of the first week the weight had fallen to 345.5 g and at the end of the second week it had reached

329.5 g or a loss in weight during the total heat exposure of 10.6%. The body temperature of the mice at the end of 2 weeks heat exposure averaged 37.69 ± 0.3°C which can be considered normal.

Discussion: An explanation of how adaptation to heat would increase anoxic resistance must include several known acclimation effects both circulatory and endocrine. Circulatory changes such as vasodilation should not prolong drowning survival unless they are accompanied by vasoconstriction simultaneously in redistributing blood to more vital areas as occurs in the seal(22) which increases oxygen economy under water. With the mouse one would look to adaptive changes in the endocrine system involving the thyroid and possibly the adrenal gland. Thyroid activity increases with cold exposure and conversely the opposite prevails under heat(20-21). With decreased endogenous heat production basal oxygen requirements fall resulting in longer survival to anoxic death.

Summary. Mice were exposed to heat in an incubator kept at 36-37°C for periods of 10 and 14 days. When removed from the incubator the mice were drowned in water of approximately body temperature and survival determined to the nearest second as witnessed by at least 2 observers. Heat adaptation increased anoxic resistance to drowning by 14.5% after 10 days and by 28.9% after 14 days. The mice kept at high temperature for 2 weeks lost approximately 10% of their weight but showed no change in body temperature.

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Improved Methods for Hemagglutination Studies with Arthropod-Borne Viruses. (21502)

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In previous communications(1,2), a method was described for conducting hemagglutination-inhibition tests with several arthropod-borne viruses. The test has since been modified to increase the stability of antigens and thus permit prolonged incubation of serum-antigen mixtures. This modification was, in part, suggested by the studies of Chanock and Sabin(3,4) on hemagglutinin of St. Louis virus. In addition, the preparation of sera has been simplified and the erythrocyte suspension standardized.

Methods. Antigens and sera. Antigens were prepared from suckling mouse brain by acetone-ether extraction as previously described(2) and usually stored lyophilized at –20°C. Sera used to obtain data in Tables II and III were prepared by Seitz filtration (2). To adjust the pH of serum in the first tube of a titration to pH 9.0, 0.05 ml of 0.3 M NaOH was added to 0.5 ml of filtered serum. The resultant slight dilution of the serum was disregarded. *Reagents.* Solutions used for tests consisted of 0.05 M borate—0.12 M NaCl at pH 9.0 and a series of 0.2 M phosphate—0.15 M NaCl adjusting diluents. Formulae for the latter are given in Table I.

Tests. Serial 2-fold dilutions (0.25 ml) of filtered sera were made in borate-NaCl, pH 9.0, and brought to 1°-2°C. Antigens were diluted at 1°-2°C in the same diluent to give 4-16 units of agglutinin/0.25 ml and this volume added to each tube. The antigen-serum mixtures were then incubated at 1°-2°C (in ice-bath in cold-room) for periods to 24 hours prior to addition of cells. The acid or neutral pH required for hemagglutination was obtained by addition to each tube of 0.5 ml of 0.25% washed chick erythrocytes in an adjusting diluent. The composition of adjusting diluent was determined by reactivity of antigen under study(2). Final cell suspensions were made at 1°-2°C just prior to use from a 10% suspension of washed cells in saline. After addition of cells, the tubes were well shaken and allowed to settle at temperature appropriate for antigen under test(2).

TABLE I. Adjusting Diluents.

	pH on adding to an equal volume of 0.05 M borate–0.12 M NaCl, pH 9.0			
	6.0	6.4	6.8	7.0
M	ml			
1.5 NaCl	100	100	100	100
0.5 Na_2HPO_4 (Dibasic)	32	102	192	252
1.0 NaH_2PO_4 (Monobasic)	184	149	104	74
Glass-distilled water	To make 1 l.			

TABLE II. Stability of Hemagglutinating Antigens.

Antigen			Reciprocal of titer after min. at 2°C				
Virus	Held at dilution	pH	0	10	30	100	200
			× 1000				
Dengue, type 1	250	6.8	4	2	2	1	1
		9	4	4	4	4	4
Japanese B	500	7	32	32	32	16	16
		9	32	16	32	16	32
Ntaya	200	7	.4	.4	.2	<.2	<.2
		9	.4	.4	.4	.4	.4
St. Louis	150	9	2.4				2.4
Uganda S	250	7	2	2	1	.5	<.25
		9	2	2	2	2	2
Yellow fever	500	9	16	16	16	16	16

Stability of antigens at pH 9.0. Comparative studies of the stability at pH 9.0 and at the pH optimal for hemagglutination were carried out with a number of agglutinins. Antigens were brought to 1°-2°C and a dilution having 4 to 16 units of antigen in 0.5 ml, on immediate titration. At intervals up to 200 minutes, titrations were done. Under these conditions, antigens of Group A, Sindbis and WEE, showed no change in titer whether held at pH 6.4 or 9.0. Table II shows the results with some antigens of Group B. No loss in titer occurred within 200 minutes when antigens were held at pH 9.0, while with most antigens an appreciable loss occurred at pH

TABLE III. Hemagglutination Inhibition.

		Antigen and length of incubation in hr											
		EEE			WEE			Japanese			St. Louis		
Serum and animal		0	4	24	0	4	24	0	4	24	0	4	24
EEE	G. pig	160*	640	2560				0	0	0			
EEE	Mouse				40	80	160						
Sindbis	”		80	80	2560	1280	1280	0	0	0	0	0	0
VEE	G. pig	80	80	80				0	0	0			
VEE	Mouse	80	80	80	20	40	40	0	0	0	0	0	0
WEE	”	40	80	160	1280	2560	5120	0	0	0	0	0	0
Dengue 1	”	0	0	0	0	0	0	80	320	1280	80	160	640
Ilheus	”	0	0	0	0	0	0	320	1280	2560	320	640	2560
Japanese	”	0	0	0	0	0	0	1280	2560	10240	640	1280	2560
St. Louis	”	0	0	0	0	0	0	640	2560	5120	1280	5120	10240
Uganda S	”	0	0	0	0	0	0	80	640	1280	160	320	1280
West Nile	”	0	0	0	0	0	0	320	1280	5120	640	640	2560
Yellow fever	”	0	0	0	0	0	0	20	80	320	80	160	320
Anopheles A	”				0	0	0	0	0	0			
GD 7	”	0	0	0	0	0	0	0	0	0	0	0	0
Mengo	G. pig							0	0	0			
Wyeomyia	Mouse	0	0	0	0	0	0	0	0	0	0	0	0

* Complete inhibition of hemagglutination with dilution of serum 1:160. 0 equals no inhibition in dilution 1:10.

6.8-7.0. In further tests, no change in titer occurred with the following antigens when held for 24 hours at 1°-2°C and pH 9.0 at a dilution containing 8 units in 0.5 ml; EEE, Sindbis, VEE, WEE, Dengue type 1, Japanese B, Ntaya, St. Louis and yellow fever.

Serum inhibition tests with long incubation. Having established that dilute antigens could be held unchanged for at least 24 hours at pH 9.0, the effect of incubation of antigen-serum mixtures was next investigated. To this end, sera were tested in triplicate against each of several antigens. One test was set up by technic previously described(2), in which serum and antigen were diluted at optimal hemagglutinating pH and cells in saline added at once without prior incubation of serum-antigen mixtures. In the second set, the antigen-serum mixtures, at pH 9.0, were held at 1°-2°C for 4 hours and then cells added in an adjusting diluent. Finally, in the third set, the antigen-serum mixtures, also at pH 9.0, were held at 1°-2°C for 24 hours before cells were added. Some results are shown in Table III.

In Table III it can be seen that when sera were tested against Group A antigens, EEE and WEE, the titers of sera after 24 hours of incubation were, in general, slightly higher than when no incubation was allowed, the difference being, in some cases, 4-fold in titer, while the non-reacting sera showed the same lack of reaction at 24 hours as at 0 time. With Group B antigens, Japanese B and St. Louis, the gain in titer was more pronounced, up to 16-fold, after 24 hours. Also, as with Group A antigens, group specificity was not affected by prolonged incubation.

Standardization of erythrocyte suspensions. Chick erythrocytes stored in Alsever's solution show a decrease in reactivity with time. Part, at least, of this decrease appears to be due to shrinkage and consequent increase in number of cells per unit volume of sedimented erythrocytes. It seemed advisable, therefore, to standardize the suspensions according to the number of cells present rather than their volume. This was done by determining the optical density of a 0.25% dilution in saline of each day's stock of 10% washed cells. Optical density correlated well with cell count without relation to shrinkage. It has been found convenient to dilute the stock suspension sufficiently to give an optical density of about 0.450 at 490 mμ as determined in a Coleman junior photoelectric spectrophotometer with a tube 10 mm in diameter. This corresponds to a red cell count of about 1.4 x 10^7 cells per cc. Such cell suspensions gave quite reproducible agglutinin titers.

Preparation of sera by Bentonite. Removal of non-specific inhibitors of hemagglutination from sera by Seitz filtration is not well suited to the preparation of large numbers of specimens. The work of Sabin and associates(5) had suggested a lipid or lipoprotein nature for non-specific inhibitors of hemagglutinins of Japanese B, St. Louis and West Nile viruses. Oker-Blom and Nikkila(6,7) had shown that treatment of serum with small amounts of clay mineral, Bentonite, resulted in a partially selective removal of lipids without a significant loss of γ-globulin. We have found that this material can be used satisfactorily to remove normal inhibitors provided certain precautions are observed. The Bentonite was U.S.P. grade obtained as a fine powder.* One per cent suspensions were prepared by sifting the powder into 0.15 M NaCl while stirring rapidly. The resultant evenly dispersed suspension showed no change in activity when stored at 4°C for weeks. Sera were diluted 4-fold with 0.15 M NaCl and treated with a volume of 1% Bentonite equal to the volume of diluted serum. The tubes were held at room temperature for 20 minutes with occasional shaking and then centrifuged for 30 minutes at 2500 RPM. Aliquots of the clear supernatant fluids were brought to pH 9.0 and a serum dilution of 1 to 10 by addition of 1/4 volume of 0.25 M borate—0.12 M NaCl pH 9.0. Sera thus treated were used to initiate a series of serum dilutions at pH 9.0 as previously described for Seitz filtered sera. In addition, 0.25 ml of each 1 to 10 dilution was mixed with equal volume of 0.05 M borate—0.12 M NaCl pH 9.0 to serve as serum control. The subsequent procedure was as described for Seitz-filtered sera. No virus agglutinin was added to serum control tubes.

Following Bentonite treatment, certain sera developed the property of agglutinating chick red blood cells. This was the only serious complication in the perfection of the method. Since the phenomenon is at least partially temperature dependent, the patterns were frequently improved by allowing tests which had been carried out at 4°C to stand at room temperature for one-half to one hour prior to reading. The problem could be completely eliminated by chilling the original Bentonite-treated serum (1 to 8 dilution) and adding 0.1 ml of 10% washed chick cells in saline per 1.0 ml of diluted serum. The tubes were in ice-bath 20 minutes with occasional gentle agitation and the cells then sedimented at 2000 RPM in a cold-room centrifuge. The clear supernatant fluids, which should show no hemolysis, were devoid of any type of non-specific activity.

Two precautions should be mentioned in using the modified method. Either freshly drawn serum or specimens stored frozen can be treated satisfactorily. However, Bentonite treatment, as described here, by no means removes all of the inhibitor from sera which have been heat inactivated (56°-60°C). In addition, following Bentonite treatment, sera should not be subjected to freezing since this leads to development of rather marked agglutinating activity for chick cells in most treated human sera. However, the simplicity of the method and its adaptability to small volumes of serum eliminates the need for prolonged storage at low temperatures.

The hemagglutination-inhibition titers obtained with the same human immune sera when treated by Seitz filtration or by Bentonite were compared. One test was carried out with a VEE antigen, typical of Group A agglutinins, and the other with an Egypt 101 strain of West Nile antigen, typical of Group B. Of 11 sera tested, the titers of 3 were the same by both methods, and the remaining ones showed 2- to 4-fold higher titers for those treated with Bentonite. This unexpected finding represents another advantage to the use of the modified procedure for serum treatment.

Summary. Modifications are described for the method of carrying out hemagglutination-inhibition tests with arthropod-borne viruses. One of the modifications is based on the stability of antigens at pH 9.0 and 1°-2°C and on the possibility of incubation of antigen-antibody mixtures for at least 24 hours. The other modification consists of a simplified method for preparing sera for the tests. The changes in technic have resulted in increase in the sensitivity of the test with no loss in specificity.

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V. Adrenal Cortical Function in Patients with Agammaglobulinemia.
(21503)

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Agammaglobulinemia, a recently recognized clinical entity, is characterized by increased susceptibility to bacterial disease, absence of gamma globulin in the serum, absence of circulating antibody, and failure of antibody production in response to antigenic stimulation. Several observations have suggested the possibility that these abnormalities in patients with this syndrome might be related to abnormal pituitary-adrenal cortical function. Wolfson, *et al.*(1) and Keidan, *et al.*(2) and others(3) reported reduced numbers of lymphocytes as a characteristic feature in their patients with agammaglobulinemia. One of seven patients previously reported from this laboratory(4) showed extreme eosinopenia in both bone marrow and peripheral blood. It has been demonstrated that both cortisone and 17-hydroxycorticosterone may produce lymphopenia(5,6), eosinopenia(6,7), inhibition of antibody production(8), decreased gamma globulin concentration(9), and enhanced susceptibility to infection(10). The purpose of the present study is to determine whether adrenal cortical function is abnormal in patients with agammaglobulinemia.

Materials and methods. Data included in this report were obtained during an intensive investigation of agammaglobulinemia in seven patients ranging in age from 4 months to 58 years. The 2 adults in this group suffered from acquired form of agammaglobulinemia and the 5 children from the congenital disease. Details concerning the clinical and laboratory studies in these patients have been published (4,11,12) or are to be reported elsewhere (13,14). In 4 of the patients, responses of circulating eosinophil count and serum 17-hydroxycorticosteroid levels to injection of ACTH were studied. In the eosinophil-ACTH response tests, total eosinophil counts were done according to the method of Randolf(15) prior to and 2 and 4 hours following the intramuscular injection of 25 mg of ACTH. Standard ACTH - 17 - hydroxycorticosteroid (17-OHCS) response tests(16) were performed employing the technic of Nelson and Samuels(17) for determination of plasma 17-OHCS concentrations before and 2 hours

TABLE I. Lymphocytes and Eosinophils in Peripheral Blood of Patients with Agammaglobulinemia.

Patient	Total leukocytes/mm^3	Total lymphocytes/mm^3*	Total eosinophils/mm^3*
E.S.	9,150	4,575	247
W.A.	24,900	4,322	170
T.A.	10,400	6,055	256
L.L.	8,950	4,654	179
F.H.	12,300	2,729	0
F.T.	4,100	2,993	246
T.T.	9,150	7,961	127

* Calculated from total white blood count and percentage of lymphocytes and eosinophils based on 200 cell differential counts.

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[†] Markle Scholar in the Medical Sciences.

TABLE II. ACTH-Eosinophil Responses in Patients with Agammaglobulinemia.*

	Eosinophil count			
Patient	Before ACTH	2 hr after ACTH	4 hr after ACTH	Max. eosinophil depletion (%)
F.H.	0	0	3	—
	0	0	0	—
E.S.	162	119	28	82
	144	137	31	79
W.A.	328	147	75	77
	167	6	27	96
T.A.	303	197	58	81

* ACTH given in dosage of 25 I.U. as single intramuscular inj.

after the intramuscular administration of 25 I.U. of ACTH. Data on normal values for these response tests have been previously published, but are included in the Tables for comparison.

Observations. In Table I are shown the total numbers of circulating leukocytes, lymphocytes, and eosinophils observed on the admission blood counts in 7 patients with agammaglobulinemia. As may be seen in the Table, none of these patients had lymphopenia and only one had an eosinopenia. Indeed, one patient had a slight lymphocytosis, despite a normal total leukocyte count. In no instance was there a coexistent lymphopenia and eosinopenia or conversely a coexistent lymphocytosis and eosinophilia.

The responses of total eosinophil count to ACTH administration in 7 tests on 4 patients are shown in Table II. With the exception of the two tests in patient F.H., whose initial eosinophil count was zero on both occasions, these responses showed a fall of eosinophils of greater than 50% of the initial value in each instance. According to the standards established by Thorn, *et al.*(18) these observations suggest that normal adrenal activity is present in at least 6 of the 7 patients with agammaglobulinemia.

Table III shows the fasting 17-OHCS concentrations and the responses of the serum concentrations of these hormones to the administration of 25 I. U. ACTH in 4 patients with agammaglobulinemia. The fasting concentrations observed in this series vary considerably but, in the group as a whole, they are neither excessively high nor excessively low. The responses of 17-OHCS concentrations to the administration of ACTH are within the normal range. The mean values for the initial concentration of 17-OHCS and responses to ACTH administration approximate closely mean values for normal persons.

Discussion. In the group of patients studied here, lymphopenia, as previously reported by others was not observed. One of these patients had a marked eosinopenia but this was not observed in other patients in this series. Fasting concentrations of 17-OHCS, the principal adrenal cortical hormones(17, 19,20,21) were not abnormal. Responses of both eosinophils and 17-OHCS concentrations to ACTH stimulation indicated a normal capacity of these patients to respond to this standard stimulation. Epinephrine-eosinophil response tests were not done since this test no longer is considered a valid criterion of adrenal cortical function(22,23,24,25).

The data presented are interpreted as evidence that there is no consistent abnormality in pituitary-adrenal function in patients with agammaglobulinemia. It appears unlikely that adrenal malfunction can be implicated as an important etiologic factor in this syndrome.

Summary. 1. Data concerning the numbers of circulating eosinophils and lymphocytes and the serum concentrations of 17-hydroxycorticosteroids in patients with agammaglobulinemia are presented. 2. Although one patient was completely lacking in circulating eosinophils and lymphocytosis was present in another, no consistent abnormality

TABLE III. ACTH-17-OHCS Responses in Patients with Agammaglobulinemia.

	17-OHCS (μg %)		
Patient	Before ACTH	2 hr after ACTH	Response
F.H.	15.8	38.6*	22.8*
	28.0	35.0	7.0
E.S.	6.5	33.7	27.2
W.A.	8.0	22.6	14.6
	18.5	46.1	27.6
T.A.	8.5	38.7	30.2
Mean	14.22	35.75	21.7
Normal mean	12.0	29.8	17.8

* 4 hr after ACTH.

in the numbers of either lymphocytes or eosinophils was observed. 3. Normal depletion of circulating eosinophils following intramuscular injection of ACTH in 4 patients with agammaglobulinemia is reported. 4. An increase in 17-hydroxycorticosteroid concentration of normal magnitude occurred in these patients in response to the intramuscular injection of ACTH. 5. The results of these investigations do not support the concept that abnormal pituitary-adrenal cortical function is a significant factor in patients with agammaglobulinemia and represent substantial evidence that the hematological disturbances observed in some of these patients are based on mechanisms other than disturbed adrenal-pituitary function.

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Metal Chelating Agents and Hepatic Cholesterol Synthesis.* (21504)

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The finding that manganese increased the incorporation of C^{14}-labeled acetate into cholesterol by rat liver *in vitro* and *in vivo* while vanadium and ferrous iron decreased this incorporation(1) suggested that metal chelating agents might also have an effect on cholesterol synthesis. The data reported here demonstrate that 8-quinolinol decreases the incorporation of C^{14}-carboxyl-labeled acetate into cholesterol by surviving rat liver while ethylenediaminetetraacetic acid (EDTA) increases this incorporation.

Methods. In vitro experiments: Liver cell clusters from young male rats were incubated with sodium acetate-1-C^{14} as previously de-

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scribed(2). Cholesterol and fatty acids were isolated and analyzed for radioactivity, also as described previously(2). The incubating medium in all cases contained 7 meq./L. of Mg^{++}, the concentration of magnesium found to be optimal for cholesterol synthesis by surviving rat liver(3). *Feeding experiments*: Young male rats were fed a diet of powdered Purina Lab Chow containing 0.1% 8-quinolinol for a period of 10 days. Then the rats were killed, the livers removed and liver cell clusters prepared. The liver cell clusters were divided into two portions, one of which was incubated with labeled acetate in a phosphate buffer(3) while the other portion was incubated with acetate in the same buffer made 0.0001M with $MnSO_4 \cdot 4H_2O$ (Table II).

Results. It is seen from Exp. 1d, Table I, that the presence of an 0.0001M concentration of 8-quinolinol in the incubating buffer markedly inhibits the incorporation of labeled acetate into cholesterol by rat liver clusters. While the concomitant presence of 0.0001M $MnSO_4 \cdot 4H_2O$ (Exp. 1e) causes no appreciable change, the presence of 0.001M $MnSO_4 \cdot 4H_2O$ (Exp. 1f) overcomes most of the inhibitory effect of the 8-quinolinol on cholesterol synthesis.

Exp. 1g and 1h demonstrate that both EDTA and EDTA, Ca salt, in 0.0001M concentration cause an increased incorporation of C^{14}-carboxyl-labeled acetate into cholesterol. A comparison of Exp. 1i with experiment 1b shows that 0.0001M $VOSO_4 \cdot 2H_2O$ in the presence of 0.0001M EDTA does not show its usual inhibition of cholesterol synthesis. On the other hand a comparison of Exp. 1j and 1c shows the inhibitory effect of 0.0001M $FeCl_2 \cdot 4H_2O$ with or without the presence of 0.0001M EDTA.

TABLE I. Effect of Certain Chelating Agents on Incorporation of $CH_3C^{14}OONa \cdot 3H_2O$ into Cholesterol by Surviving Rat Liver.*

Exp. No.	Addition of:	Cholesterol digitonide, c.p.m.	Fatty acids, c.p.m.
1a	None	526	120
b	.0001M $VOSO_4 \cdot 2H_2O$	132	62
c	” $FeCl_2 \cdot 4H_2O$	242	70
d	” 8-Quinolinol	45	56
e	” 8-Quinolinol ” $MnSO_4 \cdot 4H_2O$	83	84
f	.0001M 8-Quinolinol .001M $MnSO_4 \cdot 4H_2O$	354	129
g	.0001M EDTA	1100	158
h	” ” , Ca salt	1221	125
i	” EDTA, Ca salt ” $VOSO_4 \cdot 2H_2O$	857	178
j	” EDTA, Ca salt ” $FeCl_2 \cdot 4H_2O$	241	57

* Each pot contained 3-4 g (wet wt) of liver cell clusters, 40 ml of buffer(3) and 5 mg of $CH_3C^{14}OONa \cdot 3H_2O$ (0.3 mc/mM).

TABLE II. Cholesterol Synthesis by Excised Livers of Rats Fed a Diet Containing 0.1% 8-Quinolinol.*

Liver cell clusters prepared from:	Incubation medium	Cholesterol digitonide, c.p.m.	Fatty acids, c.p.m.
Control rats	Phosphate buffer	1318	274
Rats fed diet containing 0.1% 8-quinolinol for 10 days	Phosphate buffer	568	310
	Idem + .0001M $MnSO_4 \cdot 4H_2O$	1252	392

* Each pot contained 3-4 g (wet wt) of liver cell clusters, 40 ml of buffer(3) and 5 mg of $CH_3C^{14}OONa \cdot 3H_2O$ (0.5 mc/mM).

A number of other chelating compounds were tested for their effect on cholesterol synthesis. N, N-Dihydroxyethylglycine, β-Hydroxyethyliminodiacetic acid and nitrilotriacetic acid were found to be without effect. N-Hydroxyethylethylenediaminetriacetic acid increased the incorporation of labeled acetate into cholesterol in a manner similar to EDTA.

The effect of the chelating agents on incorporation of acetate into fatty acids is similar to the cholesterol effect in each instance but of a lower order of magnitude (last column, Table I). This finding is similar to the effect of the metals alone on fatty acid synthesis (1).

Table II demonstrates that the excised livers from rats fed a diet containing 0.1% 8-quinolinol for 10 days show a decreased ability to incorporate labeled acetate into cholesterol. The presence of $MnSO_4 \cdot 4H_2O$ in 0.0001M concentration in the incubating buffer restores the cholesterol synthesizing ability to normal.

These data would seem to indicate that 8-quinolinol depresses cholesterol synthesis by making manganese unavailable to an enzyme concerned in cholesterol synthesis. The action of EDTA is less clear, but the data suggest that it acts by preventing the inhibitory effect of vanadium on the same, or another, enzyme concerned in cholesterol synthesis. In this connection it is of interest that Mitchell has shown that EDTA prevents fatalities in mice injected with the LD_{92} dose of $NaVO_3$ (4), presumably by chelating the metal on a mole for mole basis.

Summary. 1. 8-quinolinol decreases incorporation of C^{14}-labeled acetate into cholesterol by surviving rat liver while ethylenediaminetetraacetic acid increases it.

The various chelating agents of the ethylenediamine series were generously supplied by Bersworth Chemical Co. I am indebted to Dr. Martin Rubin for helpful advice and to Mrs. Warner Wales for technical assistance.

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Chemoprophylaxis and Chemotherapy of Experimental Poliomyelitis.* (21505)

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Numerous articles have appeared on the use of chemicals for the treatment of experimental poliomyelitis in mice and in monkeys. Kramer *et al.*(1) tested 190 compounds in mice, but found none which altered the course of the disease. Powell and Culbertson(2) have reported that a mold filtrate offered some protection to mice against the MEF-1 virus, when infected peripherally. Wooley *et al.* (3) found that 4 chemicals of 98 tested prolonged the survival time in mice, infected with the Lansing strain of poliomyelitis virus. Blocking the olfactory pathway of monkeys, using a variety of chemicals, prevented the experimental disease in monkeys when the nasal route of infection was used(4,5). A new approach to the problem of chemotherapy of virus infections has been suggested by Thompson(6) using tissue culture as a chemical screening method. Brown(7) has applied the tissue culture technic in the study of effects of various chemicals in tissue culture on poliomyelitis virus. Francis *et al.*(8) have reported that fluoroacetate inhibits multiplication but does not inactivate the virus of poliomyelitis in tissue culture. They also found that this chemical alters the experimental disease in monkeys.

It is the purpose of this report to present data collected in this laboratory on chemoprophylaxis and chemotherapy of experimental poliomyelitis.

Materials and methods. Chemicals were screened in tissue culture poliomyelitis virus tests to determine inhibition of virus growth. Some of the chemicals herewith reported were screened by tissue culture technic (monkey testicular tissue) by Dr. P. K. Smith of George Washington University(9) and forwarded to us for further screening and monkey testing. He used the MEF-1 (Type 2) poliomyelitis virus, while we used the Mahoney (Type 1) virus. After a chemical was found to prevent virus multiplication in tissue culture it was then tested for virucidal activity, using mice and Lansing poliomyelitis virus. The next screening test was an *enzyme inhibition test.* The hyaluronidase-hyaluronate system was used(10) to determine the ability of the chemical to inhibit the enzyme. This system was chosen because several reports have appeared in the literature indicating that hyalu-

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ronidase may play a role in virus infections and virus spread(11,12). Since cellular enzymes may be of importance in the host-parasite relationship, we believed that preliminary screening of a given compound in an *in vitro* enzyme inactivation test may be helpful in narrowing down the number of chemicals to be tested in monkey experiments. Chemicals were tested for *toxic properties* by both feeding mice the chemical mixed in food and by oral introduction by means of a curved needle. From the maximum tolerated dose in mice, interpolation was made for the approximate dose in monkeys. The maximum tolerated dose in mg/kg of each chemical was then determined for monkeys by feeding the chemical for from 5 to 10 days. In general, cynomolgus monkeys will tolerate about two-thirds the maximum dose for mice.

Treatment of cynomolgus monkeys was carried out by introducing the maximum tolerated dose of chemical into the stomach by means of a catheter attached to a syringe. Soluble compounds were given in aqueous solutions. Insoluble compounds were placed in gelatin capsules, the capsule inserted into the cut end of a catheter, introduced into the stomach, then washed into the stomach with 5 ml of water. The *Mahoney virus* (Type 1)† was used throughout these experiments. The virus was given by stomach tube (catheter, Fr. 14) in a single dose of approximately one PD_{50}‡ (Gebhardt and Bachtold)(13). *Mother liquor* from technical gallic acid was prepared as follows: To 500 g of technical gallic acid was added 1600 ml of distilled water. This was heated and stirred until all the gallic acid was in solution. The solution was cooled to 4°C, the recrystallized gallic acid was then removed by filtering through a Buchner funnel. The filtrate represented the mother liquor. Representative chemicals of different chemical derivatives were tested for their protective effects in cynomolgus monkeys, against experimental poliomyelitis. The dosages of chemical were given generally twice daily, but in some cases more frequent administration was used. If the animals exhibited slight toxic symptoms dosages were reduced.

Results. Protective effects were obtained with chemicals in only two classification derivatives, namely *Naphtholic* and *Phenolic* compounds. In monkeys pretreated 24 hours before virus, then continued daily, with 4-amino-1-naphthol HCl, p-aminophenol, technical gallic acid, and the mother liquor of technical gallic, a fair degree of protection against the experimental infection was obtained. Not all samples of technical gallic acid, however, were effective.

When either a prophylactically active technical gallic acid preparation or its mother liquor was used therapeutically, given beginning 5 days after the virus, prevention of the disease was obtained.

Storage of mother liquor for at least 3 months seems to enhance its protective qualities. The hyaluronidase inactivating ability of mother liquor increases 10 fold in about a month of storage at 4°C. Likewise, bubbling oxygen through the mother liquor appears to alter its characteristics so that it had a protective quality, compared with the original sample (see sample mother liquor, E-3) when freshly prepared, but not oxidized.

Chemicals which show no virucidal activity but exhibit tissue culture protection and high hyaluronidase inhibiting properties appeared to have the most protective effects. A high tissue culture virus inactivating ratio (Smith)(9) does not necessarily produce protective effects in monkeys, when used as described above. The results are presented in Tables I and II.

A statistical evaluation of results is presented in Table III.

Discussion: From the results presented, particularly the protective effects of certain samples of technical gallic acid and its mother liquor, there appears to be a contaminating chemical present. Chemically purified gallic acid is non-protective. The protective chemical may be a polymer or an oxidative product responsible for the prophylactic as well as the therapeutic effects in experimental poliomye-

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† The Mahoney strain of virus was kindly supplied by Dr. David Bodian, Johns Hopkins University.

‡ One PD_{50}—dose of virus producing poliomyelitis in 50% of animals inoculated.

TABLE I. Prophylactic Treatment of Cynomolgus Monkeys with Chemicals Given Orally. Treatment began 24 hours before Mahoney virus was given orally.

Chemical used for treatment	Virucidal activity of chemical. Lansing virus in mice	Hyaluronidase inhibition by chemical, %	Dose of chemical in mg orally/kg of monkey wt. Frequency given. Treatment started 24 hr before virus	Fate of treated monkeys
Naphthols				
4-amino-1-naphthol HCl	Toxic to mice intracer.	7	208 twice daily, 18 days	2/15
Idem			830 ” ” 2 ” 415 ” ” 5 ” 208 ” ” 10 ”	0/4
Quinones				
Hydroquinone	Neg.	0	125 ” ” 16 ”	2/4
”			41, 6 times daily, 16 days	2/4
Quinhydrone	Pos.	12	50 ” ” ” 16 ”	3/4
1, 2 naphthoquinone	”	0	415 twice daily, 2 days 207 ” ” 2 ” 104 ” ” 10 ”	2/4
Phenolics				
p-aminophenol	Neg.	90	104 ” ” 18 ”	3/10
”			415 ” ” 2 ” 207 ” ” 5 ” 104 ” ” 10 ”	0/4
Gallic acid, tech. E-1*	”	100	250 ” ” 17 ”	0/12
Idem	”	100	125 ” ” 16 ”	3/6
” E-2†	”	100	250 ” ” 17 ”	5/15
” M-1‡	”	0	250 ” ” 17 ”	4/5
Gallic acid, C. P. E-A§	”	0	250 ” ” 17 ”	2/5
Idem M-A‖	”	0	250 ” ” 16 ”	2/5
Mother liquor, technical gallic acid, E-2, stored 50 days at 4°C	”	100	250 ” ” 16 ”	1/11
Mother liquor, technical gallic acid, E-3,¶ pH 3, prepared 2 days before use	Not done	100	250 ” ” 17 ”	4/5
Mother liquor, technical gallic acid, E-3, pH 6, prepared 2 days before use			250 ” ” 17 ”	4/5
Mother liquor, technical gallic acid, E-3, oxygen bubbled thru ½ hr at pH 6, then reduced to pH 3, prepared 2 days before use		100	250 ” ” 17 ”	1/5
Pyrogallol, C. P.	Pos.	0	40 ” ” 16 ”	1/5
Tannic acid, C. P.	”	49	250 ” ” 16 ”	4/5

* (E-1) = Sample #1, technical gallic acid, Distillation Products, Ind. All chemicals prepared fresh daily except where noted.

† (E-2) = Sample #2, technical gallic acid, Distillation Products, Ind.

‡ (M-1) = ” #1, ” ” ” , Mallinckrodt Chemical Co.

§ (E-A) = ” #1, gallic acid, C. P., Distillation Products, Ind.

‖ (M-A) = ” ”, ” ” , Mallinckrodt Chemical Co.

¶ (E-3) = ” #3, technical gallic acid, Distillation Products, Ind.

litis. This active principle is as yet unidentified.

Since chemicals exhibiting protective effects are non-virucidal it appears that the virus is not destroyed *per se*. Also, since protection was obtained (gallic acid, technical

TABLE II. Therapeutic Treatment of Cynomolgus Monkeys with Chemicals Given Orally. Treatment began 5 days and 7 days after Mahoney virus was given orally.

Chemical used for treatment	Virucidal activity of chemical. Lansing virus in mice	Hyaluronidase inactivation by chemical, %	Dose of chemical in mg orally/kg of monkey wt. Frequency given. Treatment started 5 and 7 days after virus	Fate of treated monkeys
Phenolics				
Gallic acid, tech. E-1; prepared fresh daily	Neg.	100	250 twice daily, 11 days; treatment started 5 days after virus	1/14
Mother liquor, gallic acid, tech. E-2; stored 3 mo 4°C	"	100	*Idem*	0/10
Idem, stored 3 wk 4°C	"	100	"	3/10
" , stored 1 wk 4°C			"	1/10
" , " " "			250 twice, one day; 300 twice, one day; 250 twice daily, 9 days; treatment started 5 days after virus	2/9
Pyrogallol, C. P.	Pos.	0	40 twice daily, 11 days; treatment started 5 days after virus	3/5
Mother liquor, gallic acid, tech. E-2; stored 3 wk at 4°C			250 twice daily, 9 days; treatment started 7 days after virus	3/8
Idem, stored 9 days 4°C			400 twice, 1 day; 300 twice, 1 day; 250 twice, 9 days; treatment started 7 days after virus	5/10
Controls (controls for Tables I and II)	Cumulative		Some sham treated with distilled water or physiological salt sol. Some untreated. Rate of infection unaffected by sham treatment	73/155 (47.1% infection)

and its mother liquor) when animals were treated beginning 5 days after virus inoculation, it would appear that the effect was not in the intestinal tract but in some area along the pathway of virus invasion. Whether the protective effect is a cellular surface blocking phenomena, or a cellular enzyme block against virus invasion or multiplication, is not known.

Summary. 1. Two of 19 monkeys (10.5%) developed poliomyelitis when treated prophylactically with 4-amino-1-naphthol HCl (Naphtholic derivative). 2. One sample of gallic acid, technical (E-1) (Phenolic deriva-

TABLE III. Chi² and Probability (P) Values.

Chemical treatment	Fate of monkeys	Chi²	P
p-aminophenol (pretreated)	3/14*	6.8	.01
4-amino-1-naphthol (pretreated)	2/19	10.9	.001
Gallic acid, tech. (E-1) pretreated	0/12	9.2	.005
" " " (E-2) "	5/15	.95	.5
Mother liquor (E-2) pretreated; stored 50 days, 4°C	1/11	6.0	.02
" " " post-treated, 5 days; stored 1 wk to 3 mo, 4°C	7/53	14.2	.001
Controls—cumulative	73/155		

* 3/14 means 3 monkeys developed poliomyelitis out of 14 inoculated with virus.

tive) prophylactically protected all of the 12 monkeys treated. A second sample of gallic acid, technical (E-2), freshly prepared, failed to exhibit prophylactic protection. Its mother liquor, however, stored for 50 days allowed only one animal of eleven to develop the disease, when used prophylactically. Of 14 monkeys treated prophylactically with p-aminophenol, 3 developed poliomyelitis. 3. When used therapeutically, both gallic acid, technical (E-1) and mother liquor (E-2) of technical gallic acid, stored at 4°C from one week to 3 months, and given to monkeys beginning 5 days after virus, 7 of 53 monkeys (13%) developed the disease, whereas 47.1% of the controls developed the disease.

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Comparison of Serum C-Reactive Protein, Glycoprotein and Seromucoid in Cancer, Arthritis, Tuberculosis and Pregnancy.* (21506)

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Serum C-reactive protein (CRP) has been reported in several clinical inflammatory states, including acute rheumatic fever, acute rheumatoid arthritis, acute infections, and myocardial infarction(1). This factor has thus been suggested as a nonspecific indicator of inflammatory reaction. An estimation of the degree of activity of these processes has been made by determination of the titer of this substance(2,3,4) in serum. Serum glycoprotein (protein bound polysaccharide) and seromucoid (mucoprotein) have also been suggested as nonspecific indicators of inflammatory processes. Elevated serum glycoproteins have also been found in pregnancy and cancer; conditions not characterized by inflammation(5). The present study compares the concentration of protein bound polysaccharide, expressed as the polysaccharide-protein ratio (PR), and seromucoid with CRP in the sera of patients having conditions in which high PR or seromucoid values were known to exist, *i.e.* arthritis, cancer, tuberculosis and pregnancy.

Methods. Patients were studied at University Hospitals and the VA Hospital. Pregnant subjects include only those in the last trimester. Normal subjects were members of the hospital personnel. The serum glycoprotein (nonglucosamine) was determined by the tryptophan method as previously described (6). In order to avoid errors due to differences in serum protein, the glycoprotein was expressed as grams of carbohydrate per 100 g of protein. Seromucoid (mucoprotein) was determined by the tryptophan method after isolation by the method of Winzler, *et al.*(7). Total serum protein and serum albumin levels

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TABLE I. Summary of C-Reactive Protein, Glycoprotein, Seromucoid, and γ-globulin Levels.

	No. of patients	C-reactive protein		Glycoprotein		Seromucoid hexose, mg/100 ml ± S.E.	γ-globulin, g/100 ml ± S.E.
		Neg.	Avg ± S.E.	mg/100 ml ± S.E.	P.R.* ± S.E.		
Normal	10	10	0	121 ± 4	1.64 ± .02	11 ± 1.0	.86 ± .06
Pregnant (last trimester)	27	17	0.6 ± .2	157 ± 4	2.41 ± .04	11 ± 0.7	.71 ± .03
Active tuberculosis	24	1	2.0 ± .2	185 ± 8	2.48 ± .09	21 ± 1.5	1.59 ± .09
Acute rheumatoid arthritis	22	1	3.3 ± .3	186 ± 6	2.55 ± .10	19 ± 0.6	1.43 ± .09
Active disseminated lupus erythematosus	1	0	4.	232	3.10	28	1.88
Carcinoma	21	3	3.4 ± .5	174 ± 7	2.90 ± .10	19 ± 2.2	1.18 ± .14
Hodgkin's disease	2	0	5	220	3.35	22	1.10
Ewing's sarcoma	2	0	3	202	2.86	18	.72
Multiple myeloma	3	1	3	217	2.63	12	.40
Agammaglobulinemia	1	0	4	161	3.01	36	.10

* Grams of hexose/100 g serum protein.

were determined by the method of Milne(8); gamma globulin, by the method of Jager and Nickerson(9). C-reactive protein (CRP) determinations were performed by the capillary precipitin method of Anderson and McCarty (2) employing commercially available C-reactive protein antiserum (Schiefflin).

Results. Results are summarized in Table I. C-reactive protein was found in the sera of 18 of the 21 patients with proven carcinomas, in 21 of 22 patients with active rheumatoid arthritis, in 23 of 24 patients with active tuberculosis, but in only 10 of 27 pregnant patients. As previously reported, elevated seromucoid levels occurred in cancer, tuberculosis, and rheumatoid arthritis, but rarely in pregnancy. Elevated serum γ-globulin concentrations were found in the tuberculosis and rheumatoid arthritis groups and to a lesser extent in the cancer group.

Results found for patients with Hodgkin's disease, Ewing's sarcoma, and multiple myeloma were similar to those noted for carcinoma. One case of agammaglobulinemia showed the presence of C-reactive protein, elevated glycoprotein, and elevated seromucoid. Correlation coefficients between CRP and glycoprotein, CRP and seromucoid, and CRP and γ-globulin were calculated for the tuberculosis, rheumatoid arthritis and cancer groups. These results are summarized in Table II. The correlation coefficient for C-reactive protein and PR was found to be significant at the 5% level for the carcinoma group. The coefficients for the same factors were significant at the 1% level for the tuberculosis and active rheumatoid arthritis groups. C-reactive protein and seromucoid were found to be significantly correlated at the 1% level in all groups. No significant correlation was found between CRP and γ-globulin.

Discussion. The consistent finding of C-reactive protein in carcinoma is of considerable interest. Hedlund(10) has reported the occurrence of acute phase protein (which appears to be similar to CRP) in 37 out of a series of 64 malignancies, using the capsular swelling technic. The finding of a higher incidence of positive reactions in our series is probably due to greater sensitivity of the precipitin test. In any event, it appears that the

TABLE II. Correlation Coefficients of C-Reactive Protein with Serum Glycoprotein, Seromucoid and γ-globulin.

	r	t	Prob.
CRP × P.R.			
Carcinoma	.480	2.26	.05
Tuberculosis	.625	3.40	.01
Rheumatoid arthritis	.566	2.90	.01
CRP × seromucoid			
Carcinoma	.645	3.37	.01
Tuberculosis	.607	3.24	.01
Rheumatoid arthritis	.784	5.43	.01
CRP × γ-globulin			
Carcinoma	–.143	0.58	0.6
Tuberculosis	.360	1.81	0.1
Rheumatoid arthritis	.354	1.60	0.2

occurrence of CRP is a common manifestation of cancer.

If one can assume that CRP always occurs with inflammatory processes, the absence of CRP in 17 of 27 pregnancies would indicate that inflammation is not characteristic of all pregnancies. By the same reasoning the consistently elevated serum glycoprotein which occurs in late pregnancy can hardly be ascribed entirely to inflammation. On the other hand seromucoid, which is elevated in active tuberculosis, cancer and active rheumatoid arthritis, but not in pregnancy, may be related only to inflammatory processes. It is noteworthy that CRP is more closely correlated with seromucoid than with glycoprotein. Although the elevation of seromucoid in carcinoma is not significantly different from that noted in rheumatoid arthritis and tuberculosis, the total glycoprotein expressed as a percentage of the serum protein is significantly higher in the carcinoma group. This indicates that carcinoma represents a state in which glycoprotein elevations may occur as a consequence of proliferative, as well as inflammatory processes. Since γ-globulin is usually associated with antibody formation, the lack of correlation of this fraction with CRP, and probably with glycoprotein and seromucoid indicates that these "acute phase protein reactants" are not indicators of antibody production. An interesting observation in this regard is the presence of a high C-reactive protein titer in agammaglobulinemia.

Summary and conclusions. C-reactive protein has been found to be present in cancer as well as in acute inflammatory states. In cancer, acute rheumatoid arthritis and active tuberculosis, CRP is significantly correlated with seromucoid. A lesser but significant correlation was found between serum glycoprotein and CRP in these conditions. In pregnancy which was characteristically accompanied by elevated serum glycoprotein and normal seromucoid concentrations, CRP was only occasionally found. This suggests that the elevated serum glycoprotein levels of pregnancy may be due to causes other than inflammation.

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Further Investigations Relative to Studies with I^{131}-Labeled Proteins, Insulin in Particular.* (21507)

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In some studies of the metabolism of insulin-I^{131} certain observations were made relative to the inclusion of inorganic iodide in washed trichloracetic acid (TCA) precipitates of whole blood. Extension of these investigations has yielded results which are pertinent to many studies involving iodine metabolism, particularly of I^{131}-labeled proteins, and are described herein. The measurement of degradation has proved to be a valuable tool in the study of insulin-I^{131} metabolism(1,3). The radioactivity of the labeled protein is originally all protein bound as measured by TCA precipitation, but shortly after injection into a rat, the tissues are found to contain a TCA supernatant fraction of radioactivity which includes degradation products of the I^{131}-labeled molecule. The rate of this change in precipitability has been taken as a measure of the rate of degradation of insulin-I^{131}. Since in liver and kidney, at least, a good portion of the supernatant radioactivity can be readily perfused out(1,2), there has been some indication that the presence of supernatant radioactivity in the blood has reflected, in part, the degradation of insulin-I^{131} in the tissues. Accordingly, the comparison of the supernatant and precipitable radioactivity in the blood has been used as one measure of insulin-I^{131} degradation. It was noted, however, that the proportions in these two fractions *in plasma* compared with the proportions in these fractions in the *whole blood* specimen from which the plasma was obtained, were markedly different. The supernatant fraction in whole blood was less, absolutely and proportionately, than that in plasma(3). This was puzzling since it has been shown that insulin-I^{131} does not enter the red blood cell (1), and it seemed unlikely that other formed elements in the blood could be responsible for the decreased whole blood supernatant fraction. Another possibility, that degradation products were precipitated by TCA in the presence of red blood cells, was therefore investigated. Inorganic I^{131} and I^{131}-labeled diiodotyrosine were studied as possible degradation products. That inorganic I^{131} is actually a degradation product of insulin-I^{131} is evidenced by the rapid increase of radioactivity concentration in the thyroids of rats given insulin-I^{131} unless the thyroid iodine uptake is blocked(1). This thyroid radioactivity is more than can be accounted for on the basis of I^{131} contamination of insulin-I^{131} (3). Further, Kallee *et al.*(4) have demonstrated, 30 minutes after the intravenous injection of insulin-I^{131} in rats, the appearance in the serum of large amounts of a radioactive substance which moved with the approximate mobility of inorganic iodide. It should be noted, however, that Haugaard *et al.*, did not find radioactivity in red blood cells (inorganic I^{131}) at reasonably long intervals following the administration of insulin-I^{131} to rats(5).

Methods and materials. In vitro studies: *NaI^{131}* was in standard solution, obtained from Oak Ridge. *In vitro* it was used in volumes of 5 to 250 λ, containing 0.01 to 0.4 μc of radioactivity. *Washed red blood cells* (washed rbcs.) were obtained by exsanguinating an adult rat, centrifuging the heparinized blood, aspirating and discarding the plasma and buffy coat, and then washing the remaining rbcs. 4 times. The rbcs. were washed each time with an equal volume of 0.9% saline which was agitated with the rbcs. before being centrifuged and decanted off. The rbcs. were made to the original blood sample volume with 0.9% saline before being used. This procedure was done at room temperature and no visible hemolysis resulted. *TCA precipitation* was performed by the addition of an equal volume of 20% TCA to the sample,

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† Research Fellow of the U. S. Public Health Service.

centrifugation after stirring, with the supernatant then being decanted off. The precipitates were washed from 1 to 3 times with 10 ml portions of 10% TCA. *Saline washes* containing radioactivity were precipitated with TCA as above, but with 100 mg of dried human plasma added as carrier protein. These precipitates were not washed. *Radioactivity of samples* was determined after they had been dissolved in 30% potassium hydroxide and brought to a volume of 30 ml. They were then counted in a well type gamma counter, and their radioactivity expressed as a percentage of the total.

In vivo studies. NaI^{131} and I^{131}-diiodotyrosine[‡] were given intravenously to adult male rats in doses of 10 μc contained in 0.5 ml. The procedure thereafter was as described elsewhere for the insulin-I^{131} work (1).

Results. Precipitation of "Degradation Products" of Insulin-I^{131} in Presence of RBC's. In experiments with insulin-I^{131}, as noted before, supernatant radioactivity was found to be less in whole blood than that in plasma. Plasma and serum, however, were found to have essentially the same partition of radioactivity. To demonstrate that the washed TCA precipitate of red blood cells contained certain components of radioactivity which alone were not precipitated by TCA, the following experiment was done: insulin-I^{131} was given to a rat which was exsanguinated 15 minutes later; the serum was obtained and divided into 2 aliquots of 1 ml each. To one aliquot of serum was added 1 ml of washed rbcs., and then both aliquots were precipitated and washed with TCA. In the aliquot to which the washed rbcs. had been added, 27% of the radioactivity was found to be in the TCA supernatant fraction. In the aliquot without rbcs., 46% of the radioactivity was in the supernatant fraction. This showed that radioactivity was definitely transferred from the supernatant to the precipitate fraction when rbcs. were present.

TCA Precipitation of NaI^{131} in Whole Blood or Plasma in vitro. As noted before, one of the degradation products from insulin-I^{131} is probably inorganic I^{131}. Accordingly, NaI^{131} was added to rat blood and plasma *in vitro* and TCA precipitation carried out immediately. The supernatant and 2 washes were assigned to the supernatant fraction. When I^{131} was added to plasma, 98.4% of the radioactivity was found in the supernatant fraction. When, however, it was added to whole blood, only 65.4% of the radioactivity was in the supernatant fraction, and 34.6% remained in the precipitate fraction. This experiment, therefore, demonstrated that I^{131} partially became a component of the TCA precipitate when added to rat blood although not when added to rat plasma.

TCA Precipitation of NaI^{131} Added to

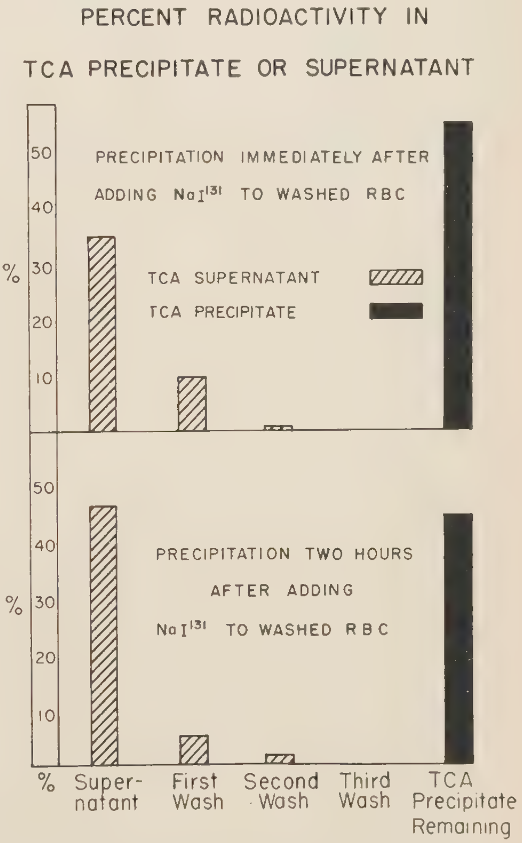

FIG. 1. NaI^{131} was added to washed red blood cells (RBC's). Trichloracetic acid (TCA) precipitation and washing done immediately (above) or 2 hr later (below). Radioactivity in each fraction is expressed as percentage of total. In both instances approximately 50% of radioactivity remained in the TCA precipitate.

[‡] I^{131}-labeled diiodotyrosine was obtained from Abbott Laboratories.

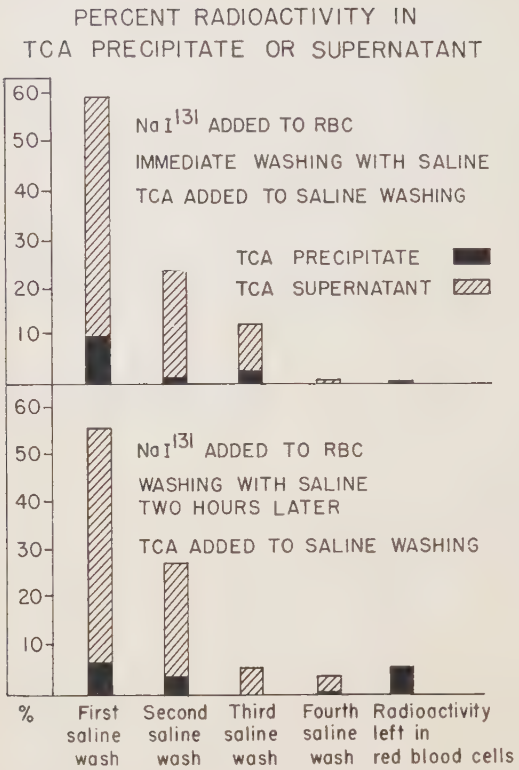

FIG. 2. NaI^{131} added to red blood cells which were then washed either immediately (above) or 2 hr later (below) with saline. Washings were then precipitated with trichloracetic acid (TCA). Little radioactivity remained in RBCs and only a minor fraction of the wash radioactivity was TCA precipitable.

RBC's. NaI^{131} was added to washed rbcs. An aliquot was precipitated and washed with TCA immediately, and a second aliquot was treated similarly 2 hours later. The precipitates were washed 3 times. The results are shown in Fig. 1. It made little difference when the precipitation was done. In the presence of rbcs., 45 to 55% of the radioactivity stayed in the precipitate.

TCA Precipitation of Saline Washes of NaI^{131}-treated RBC's. NaI^{131} was again added to washed rbcs. The cells were then washed with saline and the washings precipitated with TCA. In one experiment the saline washes were begun immediately after the NaI^{131} was added to the rbcs., and in another, the NaI^{131} and rbcs. were allowed to stand for 2 hours before the saline washings were begun. The results are shown in Fig. 2. Very little radioactivity remained with the rbcs. Even in the experiment in which the I^{131} stood for 2 hours with the rbcs., only 6% remained. Of the radioactivity in the washes, little was TCA precipitable, and it should be noted that these precipitates were not washed, so they actually contained disproportionately high concentrations of radioactivity. The I^{131}, then, could be removed from the rbcs., and once this was done, inclusion of radioactivity in the TCA precipitate was not seen.

Distribution and TCA Precipitability of Radioactivity in Rats given NaI^{131} or

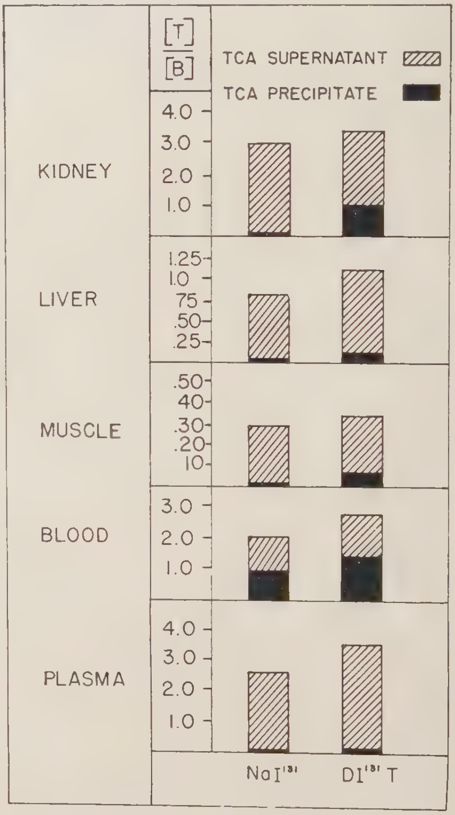

FIG. 3. Distribution of radioactivity in trichloracetic acid (TCA) precipitate and supernatant fractions of tissues 15 min. after intravenous injection of NaI^{131} and I^{131}-labeled diiodotyrosine ($DI^{131}T$) into rats. Concentration of radioactivity is expressed as $\frac{[T]}{[B]}$, the ratio of concentration in a given tissue fraction to that initially in the whole body. A value of $\frac{[T]}{[B]}$ greater than 1 indicates that concentration of radioactivity exceeds that of the whole body.

I^{131}-labeled Diiodotyrosine. With the discovery that inorganic I^{131} could be precipitated by TCA in the presence of rbcs., it was important to see if this phenomenon occurred in other tissues. The distribution and "degradation" of I^{131} was accordingly studied in the tissues of 2 adult male rats 15 minutes after the intravenous injection of NaI^{131}, using the technic used previously in studies with insulin-I^{131}(1). The results are shown in Fig. 3. A similar experiment using diiodotyrosine is also reported in the same illustration. Appreciable fractions of the latter were in a TCA precipitable form in all tissues, especially the blood. Blood was the only tissue in which significant radioactivity appeared in a TCA-precipitable form after NaI^{131} was given.

Discussion. This study has shown that at least one degradation product of iodinated proteins, namely inorganic I^{131}, in certain circumstances does not appear solely in the TCA supernatant fraction. Although in all the tissues studied except blood, inorganic I^{131} was in the TCA supernatant fraction, yet in the presence of rbcs. it could be precipitated. It has been found in this laboratory(6), that the inactivation of insulin, as measured by hypoglycemic responses in rabbits, correlated with degradation of the hormone as determined on the basis of TCA precipitability. However, from the present study, it must be stated that in the presence of whole blood a degradation product may be a non-TCA-precipitable one but, because of certain circumstances, be included in the TCA precipitate. This phenomenon can be called "re-entry"; thus, I^{131} is originally bound to insulin, and is, as such, precipitated by TCA. In the process of degradation, when free inorganic I^{131} is liberated, it is usually and appropriately in the TCA supernatant fraction and designated as a degradation product; however, in the presence of rbcs., some of this degradation product is precipitated by TCA—cannot be distinguished in our method from undegraded insulin-I^{131}—and thereby re-enters the fraction it was in originally.

The studies with I^{131}-labeled diiodotyrosine demonstrate that, should this substance be a breakdown product of insulin-I^{131} (which is not established), it too would show the phenomenon of "re-entry," and probably in more tissues than just blood.

Why this phenomenon takes place, we do not know. It is likely that in the process of denaturation and precipitation of the rbcs., inorganic I^{131} is adsorbed or occluded in the precipitate.

Summary. An appreciable portion of inorganic I^{131} was found to be precipitated by TCA in the presence of red blood cells. This was not the case with serum or other tissues studied. In evaluating the degradation of I^{131}-labeled proteins by measuring the TCA precipitability of the blood radioactivity, it should, therefore, be appreciated that the degradation product, inorganic I^{131}, may show the phenomenon of "re-entry," and be precipitated by TCA. Findings, somewhat similar to those with inorganic I^{131} were also obtained with I^{131}-labeled diiodotyrosine.

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A Simplified Colorimetric Test for Poliomyelitis Virus and Antibody.*

(21508)

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The desirability of a simple, convenient means to titrate poliomyelitis viruses and their type-specific antibodies is evident. A method requiring no unusual equipment or monkey colony could lend itself to use either by the investigator or the public health laboratory. In the simplified method herein reported, the results are indicated by color changes noted in test tubes appropriately prepared. The method owes its derivation to the following classic observations: a) Huang(1) observed in 1943 that chick-embryo tissue cultures containing phenol red indicator remained red in the presence of western equine encephalitis virus, but turned yellow if the virus was neutralized by specific antiserum. He employed other virus and tissue culture systems in a similar fashion. b) Enders, Weller and Robbins(2) made the epochal discovery that poliomyelitis virus could be propagated in cultures of various human tissues and noted color changes in the phenol red indicator under certain conditions. c) Scherer, Syverton and Gey reported that a strain (HeLa) of human epithelial cells derived from a uterine cervical malignancy could be subcultured in the laboratory "in perpetuity"(3), and that a number of viruses, including poliomyelitis, could propagate and produce cytopathic changes therein. Enders *et al.*(2) had shown that poliomyelitis viruses could be identified serologically in suspended *fragment* tissue cultures in flasks by inspection for color changes of the phenol red indicator. The results were irregular, probably because the amount of tissue present in each flask could not be readily standardized. Standardized numbers of cells for cultures became feasible with HeLa cell cultures(3,4) and with monkey kidney epithelium as employed by Dulbecco(5). Youngner's modification of the latter made possible more regularly standardized suspensions of trypsinized simian kidney cell suspensions(6). Such suspensions were utilized for titrating poliomyelitis in a color-test demonstrated to us by Youngner(6) and recently published by his group(6).

The conditions needed for using readily available reagents and a continuous tissue-culture system not requiring animal tissue were then sought and make up the body of this report.

Materials. HeLa cells growing on glass in 200 ml culture bottles and prepared according to the method of Syverton *et al.*(4)† Modified synthetic medium 199(6) adjusted to pH 7.8$\underline{\text{c}}$4.5% $NaHCO_3$. Trypsin (Difco), 1:250. Prepared as 0.5% solution in medium 199 adjusted to pH 7.8 - 8.0 filtered through paper and sterilized by passage through a porcelain filter (Selas 02). Special Metabolism Medium—(SpMM) consisting of 5% poliomyelitis antibody free monkey serum (heated at 56°C for 30 minutes) in Medium 199 with glucose added to make a final concentration of approximately 400 mg % (add 1.5 ml of 20% glucose to each 100 ml of SpMM), pH adjusted to 7.6 with 4.5% $NaHCO_3$. Penicillin and streptomycin are added to all media in final concentrations of 100 units and 100 μg per ml respectively. Poliomyelitis viruses‡. Type specific polio-

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† Supplied by Dr. R. W. Brown, George Washington Carver Foundation of Tuskegee Institute, Tuskegee, Ala.

‡ Poliomyelitis virus cultured in monkey kidney tissue culture, supplied by Connaught Laboratories as standard virus for the Poliomyelitis Vaccine Evaluation Program with the following titration data derived from trypsinized monkey kidney roller tubes:

Virus	Lot No.	ID_{50} (Avg. Score)
Type 1 (Mahoney)	54	6.8
" 2 (MEF_1)	55	6.4
" 3 (Saukett)	56	6.5

myelitis monkey antisera[§]. 13 x 100 mm Wasserman tubes (Pyrex), 00 white rubber stoppers (West Co. S 101). Phenol red pH color standards pH 6.8 to 8.0. A 0.75 ml volume of solution with trace of chloroform as preservative is heat sealed in 13 x 100 mm Wasserman tubes.

Methods. A. Preparation of HeLa cell suspension. The medium is decanted from 200 ml culture bottles as supplied, in which there are actively growing and multiplying HeLa cells. Since human serum is present in the original culture medium, washing of cells is necessary because of possible presence of antibodies to poliomyelitis virus. The cell sheet is washed once with 8 ml of plain buffered medium 199 which is then removed by decantation. Eight ml of 0.5% trypsin solution is added to each bottle. When cell sheet becomes detached from the glass, the suspension is pipetted repeatedly. This breaks up large clumps of cells to yield a suspension of single cells as well as small clumps of as many as 8 to 12 cells. The cells from several (8 to 16) bottles are pooled in a 200 ml centrifuge bottle, spun horizontally at 1000 RPM for 10 minutes. The supernatant fluid is decanted and sediment resuspended in 40 ml of the SpMM. The resulting suspension is transferred to a 40 ml tube and centrifugated at 1000 RPM for 5 minutes. This washing and centrifuging cycle is repeated twice. Finally, fresh SpMM is introduced to yield a concentration of HeLa cells of approximately 200,000 per ml (Levy or Spencer counting chamber). B. *Virus Titrations.* Decimal dilutions of virus, ranging from 10^{-3} to 10^{-8} inclusive, are prepared in SpMM. 0.25 ml of each dilution is transferred to each of eight 13 x 100 mm test tubes. To each tube is added 0.25 ml of SpMM and 0.25 ml of suspension containing 200,000 HeLa cells per ml. (See below for method of adding the HeLa cells.) C. *Serum Titrations.* Serum specimens are heated at 56°C for 30 minutes and 1:4, 1:8, 1:16, 1:64, 1:256, 1:1024 dilutions are made in SpMM and distributed in 0.25 ml amounts to each of 3 tubes. To each is added by means of a Cornwall automatic syringe 0.25 ml respectively of the three types of poliomyelitis virus diluted in SpMM arbitrarily aiming for an estimated 100 $TCID_{50}$ of virus. The tubes containing the serum and virus are shaken thoroughly, covered with a sheet of aluminum foil and incubated for at least a half-hour at room temperature. HeLa cell suspension is then added to each tube—0.25 ml of suspension containing approximately 50,000 cells—using a Cornwall automatic syringe. It is necessary to keep the cells in suspension during their distribution to the tubes. We have found it convenient to suspend a Teflon covered magnetic bar by wire and swivel joint into the flask of cells placed on the platform of a "Magne-stir" apparatus. After addition of cells each tube is tightly stoppered. The tubes are incubated in upright position at 36°C for 8 to 10 days. For convenience, sera can be diluted and stored frozen in unstoppered tubes covered with aluminum foil until enough are accumulated to make addition of virus and cells most economical of time. On day of test, the cell suspension is prepared, the three types of virus diluted for titration and for distribution to the tubes containing those serum dilutions to be tested. D. *Controls Employed.* In each day's test the following control procedures are included: 1. HeLa cell activity: To each of 4 tubes containing 0.5 ml of Sp MM are added 0.25 ml of cell suspension used in the test. Decreasing volumes of cell suspension, .20, .15, .10, .05 and .00 ml are distributed into each of 4 tubes. The volume is made up to 0.75 ml with SpMM. (Cell standardization is so satisfactory in this method that we now regard the inclusion of a range of cells from .20 ml to .00 ml as superfluous). 2. Titration of standard serums: We employ 2-fold dilutions (1:400 to 1:51,200), 4 tubes per dilution. Homologous viruses and cell suspension are added to each tube. 3. Toxicity of serums on test: To

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[§] Poliomyelitis antisera prepared by Dr. H. A. Wenner whose accompanying data follows:

50% Serum Dilution Endpoints *vs.* 100 $TCID_{50}$

Serum	In Tissue Culture
Type 1 (Brunhilde)	1:1600
" 2 (Lansing)	1:6400
" 3 (Leon)	1:6400
Normal Monkey	<1:2

TABLE I. Virus Titers *In Vitro*. Comparison of endpoints determined by color readings and cytopathogenicity, 7 tests.

Type I*		Type II*		Type III*	
Color	CP	Color	CP	Color	CP
6.8†	6.8	6.5	6.4	5.7	6.6
7.0	7.0	6.5	6.7	5.7	6.8
6.5	6.6	6.0	6.0	5.0	5.4
6.3	6.3	5.6	5.6	5.4	5.6
6.3	6.3	5.6	5.6	5.4	5.6
7.3	7.3	6.2	6.2	5.4	6.3
6.7	6.7	6.0	6.0	5.2	5.8

* Prototype virus—Connaught Laboratories.

† Calculated 50% endpoint expressed as negative $\log_{10}$.

0.25 ml of a 1:4 dilution of each serum is added 0.25 ml of SpMM and 0.25 ml of the cell suspension; no virus is added. 4. Infectivity of test dose of virus: For each of the 3 standard virus-types, 0.25 ml of the concentration selected is mixed with 0.25 ml of SpMM and 0.25 ml of cell suspension. Each mixture is tested in 4 tubes. 5. Titration of standard viruses: see (Methods—B). E. Readings. By eighth to tenth day, at which time cell control tubes have become yellow, color, or pH, readings are recorded. In serum titrations, the highest dilution of serum with a pH of 7.2 or lower (yellow-orange to yellow) is regarded as neutralization of virus and the serum titer is expressed in terms of this dilution. In virus titrations, a pH of 7.3 or higher (pink-orange to red) is indicative of presence of virus. The 50% virus infectivity endpoint is calculated by the Reed-Muench method(7). To confirm the endpoints as determined by color, tubes may be examined for destruction of the HeLa cells. In tubes where cytopathogenicity has occurred the cells no longer adhere to the glass and may be seen grossly to become separated from the wall of the tube when placed horizontally. Microscopic confirmation for the cytopathogenicity is unnecessary.

Results. A. Virus Titrations: Titration endpoints of the 3 standard viruses were calculated by the Reed-Muench method both on the basis of color and concomitant cytopathogenicity. These data are summarized in Table I. It can be seen that in 7 consecutive titrations of Type 1 virus and Type 2 virus, the endpoints by both methods agree. The Type 3 virus (which has yielded irregular infectivity endpoints in a number of laboratories)(8) yielded results with a maximum disparity in one instance of 1.1 log between the two methods of reading. Observations made during Type 3 virus titrations indicate that its cytopathogenicity appeared to be tardy. Thus in high dilution, although the virus multiplies and *eventually* destroys HeLa cells, its activity is not rapid enough to prevent acid formation by the cells. Color changes to yellow may thus occur before the cytopathogenic effects are manifest. Standard Type 3 virus (Connaught) was passaged 3 times in HeLa cells and then titrated twice by the technic described above. With this resulting pool of virus it was observed that a) its cytolytic effects appeared more promptly and b) its observed titer was higher *i.e.* $10^{-6.6}$ to $10^{-7.4}$, in distinction to the standard virus (Connaught) propagated in simian kidney cells. With our HeLa cell propagated Type 3 virus, endpoints calculated from color changes and from cytopathogenicity are in agreement.

B. *Human Serum Titrations*: Fig. 1, 2 and 3 present data comprising serum neutralization endpoints using single tubes for each dilution. These data reveal the agreements observed in endpoints determined by both methods of reading. Of 178 sera tested

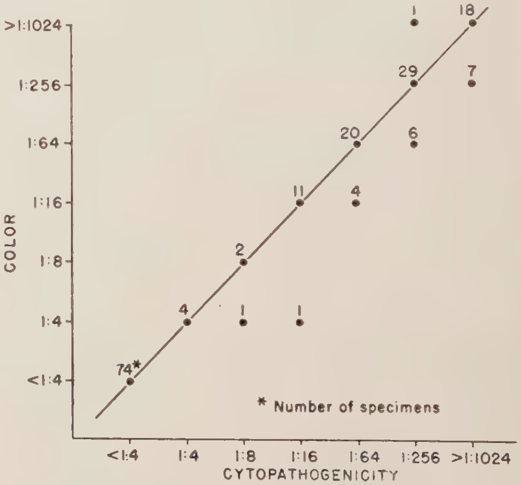

FIG. 1. Comparison by color and cytopathogenic readings of serum antibody titers *vs.* Type 1 poliomyelitis virus *in vitro*.

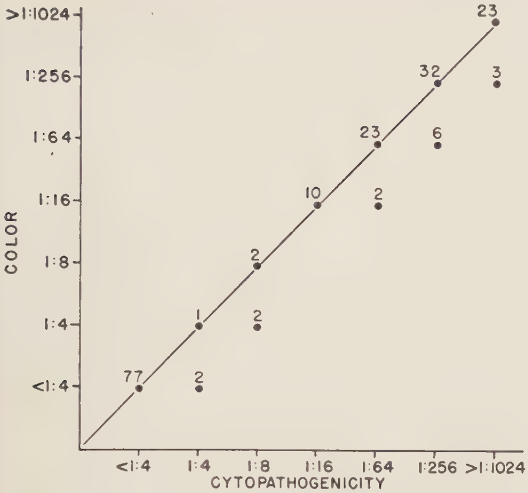

FIG. 2. Comparison by color and cytopathogenic readings of serum antibody titers *vs.* Type 2 poliomyelitis virus *in vitro.*

against Type 1 virus, 158 were in agreement by both methods; 19 differed by 1 serum dilution and only 1 by 2 dilutions.

Neutralization of Type 2 virus by the sera tested shows the same pattern as with Type 1 virus. Of 183 sera titrated, 168 agreed by both methods of reading the endpoints; 15 differed by one serum dilution, all having apparently higher neutralization titers as read by cytopathogenicity than by color endpoints.

Of 185 sera tested against Type 3 virus, 159 gave titers which were in agreement by both methods of reading the endpoint despite the erratic behaviour of Type 3 virus. Indeed the 26 discrepant instances never represented more than a difference of a single tube dilution. Seventeen of the sera titrated higher by color readings than by cytopathogenic readings. In 9 instances endpoints determined by cytopathogenicity were higher than those calculated from the color readings.

Discussion. A method has been presented for the titration of poliomyelitis virus and type specific antibodies in animal and human sera. The procedure is based on the metabolic activity of viable HeLa cells which produce acid from glucose, a reaction in which the color of phenol red is changed from red to yellow. In the presence of unneutralized poliomyelitis virus the HeLa cells are destroyed; the medium's color therefore remains unchanged.

Since the activity of cytopathogenic viruses *in vitro* is generally determined by microscopic examination for the presence of viable cells, we have used this as the criterion for determining endpoints by color changes. In the tests, herein reported, there was agreement between infectivity endpoints of Types 1 and 2 viruses as determined by concomitant readings of color changes and cytopathogenicity. Some irregularities (v. infra) in the infectivity titers of the Type 3 virus appeared, corresponding to the experience of other laboratories with this particular pool of virus (8)‖.

In antibody titrations where disparities between color (orange pink pH 7.3-7.4) and cytopathogenic endpoints were attributable to the presence of some viable HeLa cells, residual living virus was regularly demonstrated by subinoculation into fresh, stationary HeLa cell culture tubes.

Without prior knowledge of the titers determined by our method, Dr. Herbert Wenner, Kansas City(9), tested aliquots of 20 human sera against the 3 types of poliomyelitis virus, using suspended monkey cell cul-

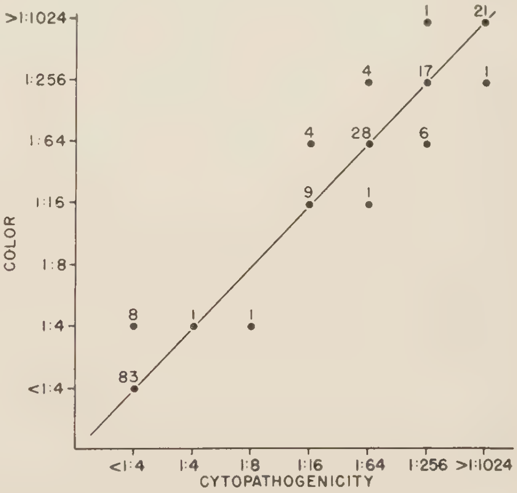

FIG. 3. Comparison by color and cytopathogenic readings of serum antibody titers *vs.* Type 3 poliomyelitis virus *in vitro.*

‖ Since completion of the manuscript 700 sera were tested against another pool of Type 3 virus with no observed irregularities.

tures in a color method(6). There was good agreement in endpoints as determined by both procedures.

The experimental conditions which affect the reproducibility of results with this method depend largely upon (1) the HeLa cell concentration used; (2) the relative alacrity of cytolytic effect of the strains of virus employed and (3) the composition and buffering effect of the nutrient utilized. In our system HeLa cells do not cause changes in the color of the medium until about 5 days after preparation of the tubes thus giving ample time for viruses to propagate and exert their cytolytic effects. Changes in the conditions could, however, result in some disparity between color and cytopathic endpoints as may occur with the tardy Type 3 virus (Connaught). In order to lengthen the time needed to produce an acid reaction in the medium, the number of HeLa cells could be decreased or the nutrient's enrichment reduced.

With viruses whose properties include rapid cytolysis, appropriate conditions for more rapid acid production (yellow color) are being established. Similar antibody observations with other viruses cytolytic for HeLa cells, are successfully in progress and will be reported separately.

The more obvious advantages of the method include: its technical simplicity, elimination of a monkey colony, relative ease of perpetual propagation of HeLa cells made into replicable suspensions as needed. Furthermore, we have observed that even low serum-dilutions do not induce toxic changes in the dispersed HeLa cells as compared with the same sera so tested in dispersed monkey kidney cells. This method lends itself to use either for large or small numbers of serum specimens on any given day.

Summary. Under the particular experimental conditions developed and herein described, the production of a yellow color by dispersed HeLa cell cultures in a medium containing phenol red, is regularly prevented by un-neutralized poliomyelitis virus. Thus, a simplified method is available for titration of the poliomyelitis viruses and their type-specific antibodies.

Addendum: Since originally submitting this manuscript, 1825 sera have been tested quantitatively against the 3 types of poliomyelitis virus by the described method.

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Propagation *in vitro* of Poliomyelitis Viruses VII. pH Change of HeLa Cell Cultures for Assay.* (21509)

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The cell culture has proved a versatile tool for the titration of virus or antibody(1,2). Monkey kidney cells or human epithelial cancer cells (strain HeLa, Gey) successfully propagated, have been the cells of choice. This laboratory has used cells of strain HeLa. Cultures of these cells in from 1 to 6 days after infection by a virus of the poliomyelitis group(2,3) or by any of a variety of other viruses(4-8), undergo rapidly progressive degeneration over a period of from 7 to 24 hours. Cessation of respiration and total destruction ensue. Homotypic neutralizing antibody entirely inhibits this fulminant destructive effect of virus, so that cellular metabolism continues and acid accumulates. The response of normal kidney cells to infection by poliomyelitis virus is essentially similar.

A colorimetric reflection of the acidity of infected cell cultures can be a useful and practical indicator of virus activity(9-11). Salk, Youngner and Ward(11) applied a colorimetric assay of poliomyelitis antibodies with monkey kidney cells, but they were unable to use HeLa cells for the purpose. Similar attempts in this laboratory to employ HeLa cells met with failure until recently(12). This report describes a method and a maintenance medium for the assay of poliomyelitis virus or antibody in HeLa cell cultures.

Materials and methods. Yeast extract medium (YEM) is a mixture of 1% Difco dehydrated yeast extract, 10.0 ml; 10% glucose solution, 3.5 ml; Hanks's balanced salt solution (H) without bicarbonate, 86.0 ml; 10,000 units of penicillin and 10.0 mg streptomycin in 0.5 ml of H. The pH by glass electrode pH meter was adjusted to 7.0 ± 0.2 by the addition of 0.6 ml of 1.4% sodium bicarbonate per 100 ml. Virus and serum samples were diluted in YEM. Cell suspensions were made in YEM, 81.7 ml; chicken serum (ChS) or monkey serum (MoS), 12 ml; and 1.4% sodium bicarbonate, 6.3 ml. The pH of the cell suspension was adjusted to 7.9 ± 0.1. If the pH exceeded 8.0, sterile 0.1N HCl was added at once to reduce the pH to 7.9. *Serum* samples were inactivated at 56°C for 30 minutes and diluted, for the rapid 6-tube screen test, to 1:4, 1:8, 1:16, 1:64, 1:256 and 1:1024. For test with the 3 types of poliomyelitis virus 0.25 ml of each dilution was distributed to each of 3 tubes. Similarly 10 successive 2-fold dilutions of serum were made in quadruplicate for more accurate antibody titrations. These dilutions could be kept satisfactorily at 4°C for 6 days before use. *Virus* of known potency was employed: the dosage in routine diagnostic tests was 100 $TCID_{50}$ per 0.25 ml; in investigative studies, 25 $TCID_{50}$. The titer was determined by transfer of serial 0.5 $\log_{10}$ or $\log_{10}$ dilutions of virus contained in 0.25 ml of YEM to 4 tubes each containing 50,000 HeLa cells in 0.25 ml YEM. These tubes and an additional 4 tubes without virus were stoppered and incubated at 37°C for 7 days. The end point was established by cytopathic effect and a distinct difference in pH between test and control tubes; the titer was expressed alternatively as the highest dilution producing these effects in 50% of the incubated tubes, or as a multiple of the 50% infectious dose ($TCID_{50}$). *Cells of strain HeLa* were cultivated in planar bottles in a medium composed of 40% human adult serum (HuS) and 60% Hanks's balanced salt solution (H), as described previously(2,3). Normal antibody-free MoS could be substituted for HuS. Immediately before use the cells on the walls of each donor bottle were rinsed 3 times with 5 ml portions of H. The bottle was revolved for thorough rinsing of the entire inner surface. For the final rinse the cells were scraped with a rubber policeman or scraper into 5 ml of H (without the aid of trypsin) and dispersed mechanically with a 10 ml pipette by alter-

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* Aided by a grant from the National Foundation for Infantile Paralysis.

nate forceful withdrawal and expulsion 10 successive times. Care was taken to avoid blowing air into the suspension since frothing resulted in loss of cells. Suspensions from the requisite number of bottles were pooled, each bottle being rinsed finally with 5 ml of BSS to harvest stray cells. The pool was centrifuged at 1500 rpm for 15 minutes; the supernatant fluid was discarded and the packed cells were resuspended in YEM-88, ChS or MoS-12, usually 5.0 ml per original bottle. An aliquot of about 1 ml was transferred to a 16 x 125 mm tube, the cells were dispersed further by use of a capillary pipette for forceful alternate withdrawal and expulsion for 10 cycles, and the cells were counted in a hemocytometer. The test cell suspension was made by dilution of the parent cell suspension with YEM-88, MoS or ChS-12 to a density of 200,000 cells per ml. Sodium bicarbonate (commonly 7.5 ml of 1.4% sodium bicarbonate per 100 ml) was added to give a pH of 8.0 ± 0.1. Finally the cell suspension was transferred to a reservoir fitted with a helical glass stirrer for distribution in 0.25 ml amounts to tubes by a Cornwall automatic pipette, as described(2).

Antibody Titration. Serum samples from patients or standard antiserums were diluted as described. Each serum dilution was mixed with 100 $TCID_{50}$ of virus, shaken thoroughly, and left for 1 hour at room temperature. Preparation of the cell suspensions and adjustment to pH 7.9 ± 0.1 must be effected immediately before dispensing the cells to forestall increase in pH over 8.0. The pH of the suspension was adjusted, as indicated. For *controls* 0.25 ml amounts of cell suspensions were added to tubes containing respectively: a) 0.25 ml of YEM and 0.25 ml YEM with 100 $TCID_{50}$ of virus (*virus control*); b) 0.50 ml of YEM (*cell control*); and c) 0.25 ml of a 1:4 dilution of serum and 0.25 ml of YEM (*serum toxicity control*). Test results were not accepted unless the virus controls at the time of reading were at a pH of 7.4 or greater and the cell controls at a pH less than 7.0. A serum toxicity control was essential for recognition of any serum sample toxic to cells. For the detection of a low antibody level in a toxic serum, the assay was repeated with cells established in culture: these cells are less susceptible to toxic agents. Since serums diluted more than 1:16 rarely were toxic, antibody titers above this level were established readily.

TABLE I. Titers of 3 Prototype Virus Pools* by Cytopathic-Established Cell Technic (C) and by pH Change-Suspended Cell Technic (P) to Show the Reproducibility of Consecutive Assays over a Period of 14 Days.

	Pool T-1		Pool T-2		Pool T-3	
	ID_{50} titer by					
	C	P	C	P	C	P
	6.6	6.6	6.2	6.6	5.7	5.5
	6.5	6.6	5.8	6.6	5.6	5.4
	6.8	6.6	6.4	7.2	5.5	5.5
	6.8	6.8	6.0	6.5	5.8	5.8
	6.6	6.6	5.8	6.8	5.7	5.6
Arithmetic mean	6.7	6.6	6.0	6.7	5.7	5.6

* Standard control poliomyelitis viruses provided by Connaught Medical Research Laboratories for the Poliomyelitis Vaccine Evaluation program.

TABLE II. Results of Successive Antibody Titrations of Standard Poliomyelitis Antiserums* by Cytopathic-Established Cell Technic and by pH Change-Suspended Cell Technic.

	Poliomyelitis antiserum		
	Type 1	Type 2	Type 3
pH change-suspended cell technic	1600	1600	6400
	”	6400	”
	”	1600	”
	6400	6400	”
	1600	”	1600
	”	”	6400
	”	25,600	”
	”	1600	”
	”	6400	”
Cytopathic-established cell technic	”	”	”
	”	”	”
	”	1600	”
	”	6400	”

* Standard type specific antiserums supplied by Dr. H. A. Wenner for the Poliomyelitis Vaccine Evaluation Program.

Results. Data supporting the applicability of the procedure are contained in Tables I-III. Parallel titrations of virus or antibody were carried out with established cells by conventional technic(2), and with suspended cells by the procedure outlined. The two technics, as applied to the poliomyelitis vaccine evaluation study, yielded reasonably well correlated results; in the replicate antibody assays performed (Tables II, III) differences

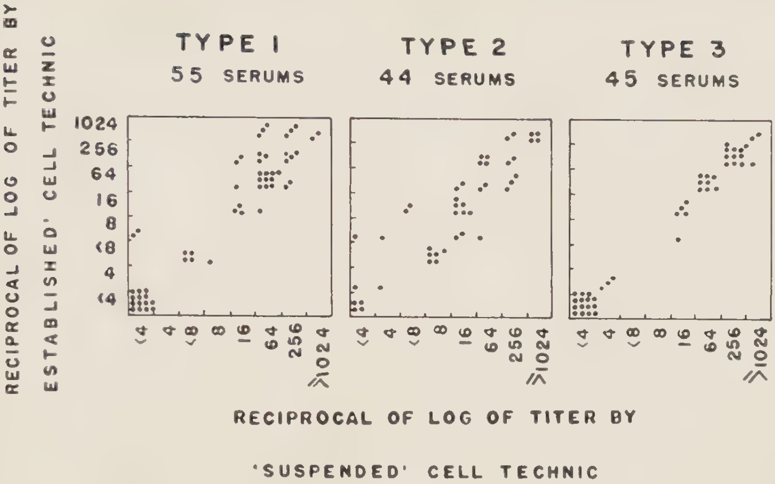

TABLE III. The Results of Comparative Assays of Poliomyelitis Antibody by 'Established' and 'Suspended' Cell Technic.

exceeding the expected ± 4-fold error were not encountered.

Discussion. The reported method for assay of poliomyelitis virus or antibody utilizes as indicator acid production by HeLa cells in culture: infectious virus destroys the cells before metabolism materially lowers the pH of the culture medium while antibody neutralizes virus and permits continued acid production with change in the color of the medium to yellow. Success depended on the use of yeast extract medium at a pH of 7.9. This medium permitted survival of the cells over a pH range from 8.0 to 6.6; in 4 to 6 days, a period sufficient for infection by poliomyelitis virus, the acids produced by uninfected cells turned the phenol red indicator yellow.

Actively metabolizing cells were essential. Bottles containing 3 to 7 million cells fed within 48 hours before use by 2.0 ml of human or monkey serum provided satisfactory suspensions. The variable capacity of chicken serums to complement yeast extract made pools desirable; a months' supply of pooled serum could be kept at 4°C. Antibody-free monkey serum was superior to chicken serum for maintenance of HeLa cells under suboptimal conditions; additional bicarbonate was required to compensate for the acceleration in cellular metabolism during the first two days of incubation. For emergency elevation of the pH from 7.4 to 7.7 the stoppers were released or removed for one hour. The open tubes were protected with aluminum foil to prevent contamination.

The use of yeast extract and the formula for YEM were derived after assorted maintenance solutions containing various buffers and varied amounts of glucose had yielded equivocal findings. The yeast extract served as a rich source of B vitamins, amino acids, and possibly other growth factors as yet unidentified.

The merits of the procedure for assay of poliomyelitis virus and antibody are: a) yeast extract medium is made from materials quickly available and economically purchased; b) cells are washed free of antibody while still attached to the glass surface of the donor bottles so that tedious washing of large numbers of individual tube cultures is avoided; c) savings in time and glassware are conserved by transfer of cells directly to tubes containing incubated virus-serum mixtures, providing tube cultures while eliminating the need for large numbers of additional pipettes for transfer of the incubated virus-serum mixtures to prepared established cell cultures; d) the endpoints of titrations of poliomyelitis virus or antibody are established by inspection with assurance that results can be confirmed by microscopic examination of the tubes.

Summary. A method is described for use of suspended HeLa cells and phenol red pH indicator for assay of poliomyelitis virus or antibody. A simple medium containing yeast extract and 4% chicken or monkey serum is used to maintain the cultures during incubation with virus or virus-serum mixtures. Infectious virus is revealed by unchanged color of the medium over a 6 day incubation period; neutralization of virus by antibody permits normal cellular metabolism during this period to produce acid with a change in color of the phenol red indicator in the medium to yellow. Evidence is presented for the agreement of results obtained by this method with those of the conventional established cell technic.

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Skeletal Growth of the Rat.* (21510)

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For a thorough understanding of the dynamics of calcium and phosphorus metabolism, it is necessary to know the normal growth pattern of the skeleton which contains more than 99% of the total body calcium(1) and from 65% to 93% of the body phosphorus (2,3). Zucker and Zucker showed that, in rats, a simple relationship existed between femur weight and body weight(4). When the log of the body weight is plotted against the log of the femur ash weight, a linear curve is obtained. However, except for a similar relation between femur length and age(5), this finding has not been confirmed for other strains of rat; nor was it extended to include other skeletal components. The studies reported here were instituted to determine to what extent the findings of Zucker and Zucker could be generalized: if the skeletal weight could be correlated with age as well as body weight and, in particular, if this treatment of data would be applicable to the Rochester strain of rats (derived from Wistar).

Methods. Groups of both male and female rats (totaling 225) of the Rochester strain were sacrificed by decapitation at varying ages from 15 to 170 days of age. The skeleton of the male rats was separated from the soft tissues by autoclaving at 15 lb pressure for 2-15 minutes the carcasses from which the viscera had been removed. The skeletal components, long bones, skull and 4 lumbar vertebrae, were then separated, dried and ashed at 750°C overnight. The female rats were eviscerated and the femora were removed. The remainder of the carcass was ashed at 475°C overnight. The ash from soft tissues by this procedure is a soft powder and can be separated by gentle sifting through 10, 20, and 80 mesh screens successively. Following

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* This paper is based on work performed under contract with the U. S. Atomic Energy Commission at the University of Rochester Atomic Energy Project, Rochester, N. Y.

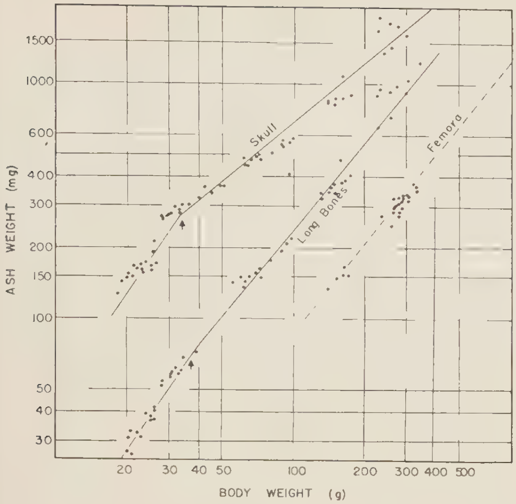

FIG. 1. Graphic representation of relationship between body wt and skeletal wt of the male rat. The dashed line for the femora is taken from Zucker and Zucker(4).

this initial separation, the partially ashed skeletal tissue is reheated to 750°C for 4 hours for the final ashing.

Results. As has been known, the rate of growth of the male rat is greater than that of the female. Further, the rates of growth of the several skeletal components vary within one sex and vary with comparable bones of the two sexes. Thus, it is evident that while the study of a given bone may give data qualitatively representative of the skeleton as a whole it will not give a quantitatively accurate picture of the skeletal metabolism of calcium or phosphorus.

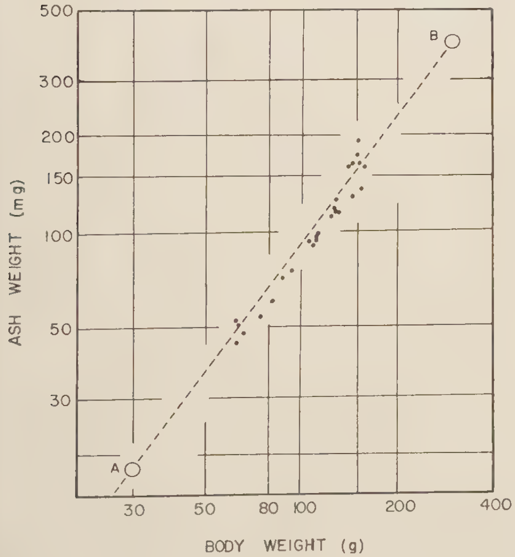

FIG. 2. Relation between femur ash wt and body wt. Data of Zucker and Zucker(4) are compared with linear relationship (dashed curve) obtained in the present study. A is the point near the age at weaning and B the point where the curves for male and female animals diverge.

Fig. 1, 2, and 3 are plots of the data presented in the manner of Zucker and Zucker (2), showing that their type of representation is applicable to the Rochester strain of rat and to various bones of the skeleton. Not only are the curves linear, but the slope of the line for the female femora, 1.34, (Fig. 2) compares with a slope of 1.347 as found by Zucker and Zucker for the same bone. These data also show the variation in growth rates of the several bones, as seen in the slopes of these lines (Table I). Once again the change in these body weight-bone weight relationships which was reported by Zucker and Zucker(4) is seen at the age of weaning by the distinct breaks in the lines in Fig. 1.

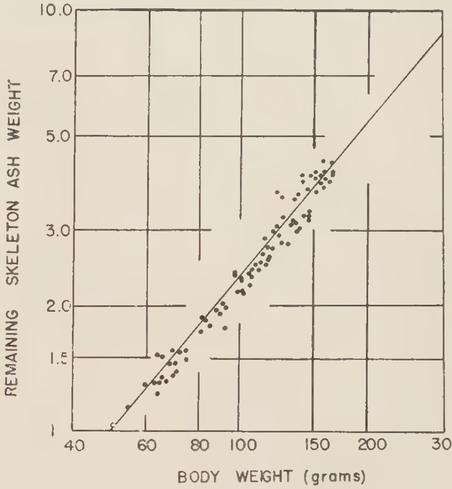

FIG. 3. Relationship between skeletal (total skeleton minus the femora) wt and body wt.

From the data presented it is seen that a linear relationship exists between the body weight and the femur ash weight, and between the body weight and the total skeletal ash weight on the log-log plot. Therefore there will be a similar relationship between femur

TABLE I. Comparison of Slopes ("d" and "c" of Equations 1, 2, and 3) Obtained by Log-Log Plot of Ash Wt *vs.* Body Wt.

	Component	Slope (this study)	Slope (Z and Z) (4)
♂	Femora	1.28	1.282
	Longbones	1.27	—
	Skull	.95	—
	Vertebrae	1.41	—
♀	Femora	1.34	1.347
	Total remaining skeleton	1.23	—

ash weight (or any other single component) and total skeletal ash weight. That is

$$y = ax + b \quad (1)$$

$$z = cx + d \quad (2)$$

in which x = log body weight; y = log total skeletal ash weight; z = log femur ash weight; and a, c, b, and d are the appropriate slopes and intercepts, and

$$y = (a/c)z + b - a\frac{d}{c} \quad (3)$$

The equation which is applicable to the components studied here would presumably hold for all skeletal components. Thus all that would be required for the establishment of the comparative growth rates of any two bones would be weights at 2 different ages (post-weanling).

It is important to note that this equation is similar in form to equations derived for the growth of body organs other than the skeleton (5).

Summary. The applicability of a graphical presentation of skeletal weight data as a function of body weight on log-log graph paper to the Rochester strain of rat and to individual components of the skeleton has been demonstrated. It has also been shown that the function relating the two variables is the same as for a previously described colony. Further, the variations demonstrated among the components of the skeleton preclude the use of any one bone as quantitatively representative of the skeleton as a whole. However, an equation for comparing the growth rates of the different bones, which require only the weights at two ages, has been proposed.

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Phospholipid Fractions in Multiple Sclerosis and Normal Serum.* (21511)

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While histological technics leave no doubt as to the highly lipid character of the myelin sheath, insight into the chemical nature of these lipids has been derived mainly from comparative analysis of white and grey matter, and from measurements of lipids in nervous tissue during periods of active myelin deposition. In a study of the former type, Johnson, McNabb and Rossiter(1) found a higher concentration of phospholipids, particularly sphingomyelin, cholesterol, and cerebrosides in white matter. Studies of the latter type (2-4) lead to the conclusion that myelin deposition is correlated with an increase in phosphatides and cerebrosides.

The process of demyelination has been evaluated almost entirely on an histochemical basis, and it is generally accepted that this involves a loss of lipids in the myelin and its

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* This work aided by grant from Multiple Sclerosis Foundation of America, Chicago. Dr. Lewis J. Pollock, responsible investigator.

replacement by glial tissue, largely of non-lipoidal character. Thus in a demyelinating process, there is a loss of lipid material, including phosphatides and cerebrosides, from the myelin sheath, and a study of these components would be desirable in such a disease process. Since the amount of cerebrosides, if present, would be undetectable by current analytical technics in the amount of blood that could be reasonably obtained(5), this study is concerned with the total phospholipid, lecithin, cephalin and sphingomyelin concentrations in the serum of patients with multiple sclerosis.

Methods. Extraction of phospholipids was accomplished by adding 6-8 ml of serum to 20 volumes of alcohol-ether(3:1), which was then brought to boiling in a water bath, and allowed to cool for 1 - 2 hours. The extract was filtered into a 200 ml volumetric flask with washing, the residue re-extracted with ether in a Soxhlet for 6 hours and the extracts combined and made to volume. An aliquot was removed for phosphorus determination, the remaining extract was reduced to 0.5-1.0 ml under nitrogen *in vacuo,* the phospholipids dissolved in anhydrous petroleum ether, and the residue extracted with water. The petroleum ether extract was made up to 50 ml, an aliquot removed for phosphorus analysis, and the remaining extract treated with MgO. The water extract was used *in toto* for analysis. The separation of the phospholipid fraction into choline and non-choline phosphatides was carried out according to the method of Taurog *et al.*(6), where the phosphatides are adsorbed on MgO. Lecithin and sphingomyelin are then eluted with methanol, while cephalin remains on the adsorbent. The separation of lecithin and sphingomyelin was performed according to Schmidt, *et al.*(7), where the phosphatides are incubated with N/1 KOH at 37°C for 24 hours. Under these conditions, sphingomyelin is alkali-stable, lecithin alkali-labile, and thrichloracetic acid is then added to precipitate the former. Phosphorus was determined according to Gomori(8). Sulfuric acid has been used to digest the extracts, except in the determination of sphingomyelin where $HClO_4$ was employed to avoid salt formation. An excess of $HClO_4$ was avoided to obviate interference in subsequent color development.

TABLE I. Reproducibility of Results on Pooled Serum Sample.

	mg % phospholipid-P			
	1	2	3	4
Total phospholipid-P	8.77	8.80	8.70	8.63
Petroleum ether extract	8.20	8.08	8.08	8.10
Cephalin	.37	.49	.35	.38
Lecithin	5.76	5.59	5.73	5.72
Sphingomyelin	2.07	2.00	2.00	1.98

Cephalin – P was calculated as the difference between the petroleum ether extract – P and the methanol eluate – P. Lecithin phosphorus and sphingomyelin phosphorus were determined directly, but since this accounted for all of the phosphorus present in the methanol eluate, the data presented are the sphingomyelin values as calculated by difference between the methanol eluate – P and lecithin – P. Pathological serum was obtained by withdrawing approximately 25 ml of blood from patients with a diagnosis of multiple sclerosis established by the neurological consultant staff at this hospital. Patients in both exacerbated and quiescent states were selected at random, except that the age group was limited to under 40 years. Since no differences were apparent between the active and non-active states, the data were combined. Normal subjects were obtained from the laboratory and resident staff. All determinations were performed on the fasting specimen.

Results. To test the reproducibility of the method, a pooled serum sample was divided into 4 aliquot parts and determinations carried out in duplicate, as described. The results are shown in Table I, and indicate that the method is reproducible and consistent with analytical standards for accurate determinations in biological material.

TABLE II. Recovery of Lecithin and Cephalin.

	mg phospholipid-P			
	mg added	mg adsorbed	mg eluted	Recovery, %
L-α-dimyristoyl lecithin	.230	.230	.231	100
	.158	.158	.158	100
L-α-dimyristoyl cephalin	.113	.113	.012	10.6

TABLE III. Phospholipid Fractions in Normal and Multiple Sclerosis Serum.

	(1) Total phospholipid-P, mg %	(2) Cephalin-P		(3) Lecithin-P		(4) Sphingomyelin-P	
		mg %	% of (1)	mg %	% of (1)	mg %	% of (1)
Normal (14)	9.92 ± 1.27*	.82 ± .38	8.4 ± 3.6	6.45 ± 1.08	65.0 ± 5.8	1.95 ± .43	19.7 ± 3.6
Multiple sclerosis (17)	8.86 ± 1.52	.86 ± .49	9.7 ± 5.2	5.69 ± 1.21	64.2 ± 7.6	1.60 ± .49	18.0 ± 3.7
t	2.09			1.98	—	2.09	—
P	.05			.06		.05	—

* Stand. dev.

To determine the validity of the MgO separation, synthetic L – α dimyristoyl lecithin and L – α dimyristoyl cephalin† were dissolved in benzene, the compounds adsorbed on MgO, and eluted with methanol. The results appear in Table II, and show complete adsorption of both compounds by MgO, since analysis of the solvent after MgO treatment showed a complete absence of phosphorus. On elution with methanol, the entire lecithin fraction appeared in the eluate. However, contrary to theoretical expectations, 10.6% of the cephalin fraction could also be eluted. Since the preparation was one compound, and not a mixture customarily encountered in biological tissues, the partial elution of the material could be explained by its contamination with degradation products. This was confirmed by Dr. Baer.‡ It was concluded therefore that methanol would not elute cephalin from a MgO adsorbent.

The average values, standard deviations and statistical analysis of 14 normal and 17 multiple sclerosis sera are presented in Table III. The total phospholipid-P was 9.92 mg% and 8.86 mg% for normal and pathological groups respectively (t = 2.09, P = .05). In both groups the average cephalin values showed a striking similarity which was not apparent for the other fractions, averaging .82 and .86 mg% in the two groups. The normal values are in agreement with those reported by Sinclair(9) and Hack(10). This diminution in the phospholipid fraction of the multiple sclerosis group was accounted for chiefly by the lecithin component which was 5.69 mg % as compared to 6.45 mg% in the normal subjects (t = 1.98, P = .06). Despite this decrease the lecithin fraction was a constant component of the total phospholipids, averaging 65.0% in the normal group and 64.2% in the pathological, although lecithin variations from 3.12 mg% to 8.28 mg% occurred. Sphingomyelin values also showed a trend in the direction of lower values in the multiple sclerosis patients with values of 1.60 mg% in this group as compared to 1.95 mg% in the normal subjects (t = 2.09, P = .05). This constituted 18.0% and 19.7% of the total phospholipid fraction of the respective groups. The normal values are in agreement with those found by Taurog, Entenman and Chaikoff(11).

The petroleum ether insoluble residue averaged about 6 - 7% of the alcohol:ether soluble phosphorus in both groups. This residue was completely recovered by dissolving in water. The occurrence of this insoluble material has already been noted by Folch and Van Slyke (12), who remarked that the residue was suggestive of sphingomyelin, but its solubility in water would preclude such a possibility. A more likely explanation would reside in the hypothesis that this fraction constitutes an inositol phosphatide, although such a component has not been demonstrated in human serum as yet.

Discussion. Chiavicci and Sperry(13) reported total phospholipid values in multiple sclerosis and control groups very similar to the values reported in this study. It was their impression that the phospholipid values in the patient group were significantly lower than in the control population. The two studies differ in that the controls of Chiavicci and Sperry were patients with nervous system pathology other than multiple sclerosis, whereas normal subjects were used in this work.

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† We are indebted to Prof. Erich Baer of the University of Toronto for his generous gift of these compounds.

‡ Private communication.

A diminution of phospholipids in the blood would be contrary to expectations if this merely reflected an absorption of the degradation products of myelin degeneration. That such an absorption could take place is indicated by the work of Sweet and Locksley(14) who found that molecules as large as proteins are absorbed from the subarachnoid space. It remains to be determined whether or not a disturbance of phospholipid metabolism in the central nervous system may affect the phospholipid content or turnover of the blood plasma.

Summary. Determinations on the serum of normal and multiple sclerosis serum for cephalin, lecithin and sphingomyelin, showed normal values for these fractions of 9.4%, 65.0% and 19.7% of the total phospholipids. The multiple sclerosis group exhibited a similar distribution of these components, though the total phospholipids tended to be lower than in the control group.

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IV. Effect of O-Diazoacetyl-L-Serine (Azaserine) on Growth of Various Mouse and Rat Tumors.* (21512)

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O-diazoacetyl-*L*-serine (azaserine)—a new antibiotic isolated from the culture media of a *Streptomyces*(1)—inhibits the growth of Sarcoma 180 in mice(2). It was therefore of interest to study the effect of this agent on other mouse and rat tumors. The present communication describes the results.

Materials and methods. The tumors used in the present study are listed in Table I. The methods employed in the cancer chemotherapy studies have been described before (3). In general, subcutaneous implantations of small pieces of tumor, each measuring about 1.5 mm in any dimension, into healthy young animals (18 to 22 g mice; 100 to 125 g rats) were carried out by the usual trocar method. In each set of experiments, tumor-bearing animals were divided into two groups, one for treatment with preparations of the antibiotic and the other for controls. The progress of the tumors in the animals was re-

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TABLE I. Effect of Azaserine on Various 1-Day-Old Mouse and Rat Tumors. (Dose: 5 mg/kg/day.)*

	No. of deaths		Avg wt change in g, treated/controls†	Result of treatment	
	1st wk	2nd wk		1st wk	2nd wk
Mouse tumors					
Sarcoma 180 (solid)	1/70	2/70	0 /+2	++	+
" " (ascites)	0/40	4/40	+0.5/+6.5	++	+
" T 241	0/50	1/50	0 /+1	±	±
" MA 387	0/30	0/30	—0.5/+1.5	—	—
Adenocarcinoma E 0771	0/60	0/60	—0.5/+1	+	+
Bashford carcinoma 63	0/40	0/40	0 /+3	±	—
Miyono adenocarcinoma	0/40	1/40	—0.5/+1	±	±
Ehrlich carcinoma (solid)	0/30	1/30	0 /+3.5	±	±
" " (ascitic)	0/50	8/50	+0.5/+4.5	++	+
Krebs 2 carcinoma (ascites)	0/25	3/25	0 /+6	+	+
Carcinoma 1025	1/50	2/50	—2 /+1.5	±	—
Grand epidermoid carcinoma	0/25	0/25	—1.5/+1.5	±	±
Wagner osteogenic sarcoma	0/40	3/40	—1.5/+2	—	±
Ridgway osteogenic sarcoma	0/40	1/40	—1.5/+2	—	—
Patterson lymphosarcoma	1/25	2/25	—1.5/+1.5	+	+
Mecca "	0/60	1/60	—1 /+2	++	+
Gardner "	0/40	0/40	—0.5/+1.5	±	±
Harding-Passey melanoma	2/55	7/55	0 /+2.5	—	±
Lewis bladder carcinoma	0/25	2/25	—1 /+1	±	—
Lewis lung "	0/20	1/20	0 /+1	—	—
Rat tumors					
Flexner-Jobling carcinoma	2/70	16/70	+3 /+22	+	±
Walker carcino-sarcoma 256	3/80	22/80	—5 /+27	++	++
Sarcoma R39	1/15	3/15	—6 /+15	+	+
Jensen sarcoma	1/35	5/35	—5 /+16	++	+
Murphy-Sturm lymphosarcoma	1/70	16/70	—3 /+21	++	+

* Five or 10 animals in each test group. Results presented are avg of combined groups.

† After one wk treatment.

corded graphically after measuring them in 2 diameters with calipers 1, 2, 3, 4, and 6 weeks after tumor transplantation. In the case of ascites tumors(4), intraperitoneal injection of 0.1 cc of the fluid containing about 1 million cancer cells was made in the inguinal region with a ¼ cc syringe and a 23-gauge needle. The animals were given food (Purina Laboratory Chow) and water *ad libitum*. The first intraperitoneal injections of the crude culture filtrates or saline solutions of azaserine were given one day after tumor implantation and injections were repeated each day for 7 days. The *degrees of inhibition* of solid tumor growth were graded according to the following scheme: 1) — No effect, when average diameter was ¾ or more of average diameter of control tumors. 2) ± Slight inhibition, when average diameter was ½ to ¾ of average diameter of control tumors. 3) + Moderate inhibition, when average diameter was ¼ to ½ of average diameter of control tumors. 4) ++ Marked inhibition, when average diameter was ¼ or less than that of control tumors.

Degrees of inhibition of ascites tumor growth were graded according to the following scheme: 1) No effect (—) indicates marked abdominal distention, 4 or more times normal gain in body weight of normal mice. 2) Slight inhibition(±) indicates moderate abdominal distention. 3) Moderate inhibition (+) indicates slight abdominal distention, 2 times normal gain in body weight. 4) Marked inhibition (++) indicates no abdominal distention, or normal gain in body weight, about 0.5 g/day. 5) Survival of more than 50% of treated animals beyond 3 weeks was considered as a marked effect. Control animals with ascites die in 2 to 3 weeks.

Results. While attempts to isolate the active principle in crystalline form were progressing at the Parke-Davis laboratories(1), crude culture filtrates of *Streptomyces fragilis* were studied for their effects upon the growth of several transplantable mouse and rat tumors. These studies were followed with tests of crude preparations containing azaserine and eventually of crystalline natural or synthetic azaserine.

From Table I it can be observed that azaserine isolated from the culture filtrates is capable of inhibiting some of the tumors. Those which were found sensitive are Sarcoma 180, Adenocarcinoma E 0771, Patterson lymphosarcoma, and Mecca lymphosarcoma, as well as the 3 ascites tumors studied. Other mouse tumors tested were not inhibited by azaserine. The results with crystalline azaserine agreed well with those from the crude preparations tested at levels of comparable azaserine content. Azaserine at a dose of 2.5 mg/kg/day, corresponding to one-half of the maximum tolerated dose, had only a slight inhibitory effect on all rat tumors. It is interesting to note that in the case of rat tumors azaserine produced greater damage to tumors than the crude filtrates or partially purified materials at comparable dose levels. The latter had practically no inhibitory effect on rat tumors.

The success of azaserine in producing damage in 1-day-old tumors of the mouse and of the rat led to its test on 7-day-old tumors. Seven daily treatments with this substance produced no effect on any of the tumors used in the present study with the exception of Sarcoma 180. All tumors grew practically normally.

In the total control group of 1,500 mice and 500 rats implanted with various mouse and rat tumors, the transplants took in 100% of the cases, and the per cent tumor regression was as follows: Sarcoma 180, 5; Sarcoma T241, 0; Sarcoma MA 387, 2; Adenocarcinoma E 0771, 1; Bashford carcinoma 63, 6; Miyono adenocarcinoma, 2; Ehrlich carcinoma, 0; Carcinoma 1025, 2; Grand epidermoid carcinoma, 30; Wagner osteogenic sarcoma, 5; Ridgway osteogenic sarcoma, 7; Patterson lymphosarcoma, 3; Mecca lymphosarcoma, 2; Gardner lymphosarcoma, 1; Harding-Passey melanoma, 6; Lewis bladder carcinoma, 0; Lewis lung carcinoma, 2; Flexner-Jobling carcinoma, 15; Walker carcinosarcoma 256, 3; Sarcoma R 39, 6; Jensen sarcoma, 6; and Murphy-Sturm lymphosarcoma, 5.

Summary. 1. The effects of crude culture filtrates of a *Streptomyces,* of partially purified and of crystalline preparations of azaserine have been tested against a spectrum of 17 tumors of the mouse, 5 tumors of the rat and 3 ascites tumors of the mouse. The effects of crude and partially purified filtrates of P165 on these 17 mouse tumors were generally similar to those of crystalline azaserine. 2. With mouse tumors one day old at start of experiment, daily maximum tolerated doses of 5 mg/kg/day of crystalline azaserine had a marked inhibitory effect on Sarcoma 180; a moderate inhibitory effect on Adenocarcinoma E 0771, Patterson lymphosarcoma and Mecca lymphosarcoma, and on Sarcoma 180 ascites tumor, Ehrlich ascites carcinoma and Krebs 2 ascites carcinoma; 13 other tumors have been either only slightly inhibited or not affected. 3. There was no inhibition of any of the tumors except Sarcoma 180 when tests were made with seven-day-old growths. Daily doses of 5 mg/kg/day of crystalline azaserine in rats had a definite inhibitory effect on Walker carcino-sarcoma 256, Sarcoma R39, Jensen sarcoma and Murphy-Sturm lymphosarcoma, but only a slight inhibitory effect on Flexner-Jobling carcinoma.

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Quantitative Effects of Corticosterone on Rat Bone Marrow. (21513)

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Few attempts have been made to apply quantitative methods to enumerate bone marrow cells. The counting of these cells in tissue sections(1-3) is excessively time consuming and samples only a small part of the bone marrow. Moreover, methods previously described for the determination of the marrow mass within a bone(4,5) have necessitated destroying the bone marrow. Some workers (6-8) have computed concentrations of bone marrow cells using blood pipets. Recently, (9) a technic has been presented which involves the determination of the cellular types and numbers in a weighed sample of bone marrow.

The present method is rapid, simple and furnishes an estimate of the total amount of rat femoral bone marrow. This experiment describes the effects of corticosterone on blood formation by the marrow. This steroid was used because it has been reported to be a major adrenal cortical secretion of the rat(10).

Materials and methods. Six intact male rats of a modified Long-Evans strain and weighing approximately 250 g were injected daily for 2 days with 4 mg corticosterone suspended in 1 ml 0.9% NaCl. Adrenalectomy was performed on the third day. Similar subcutaneous injections were continued on this day and for the subsequent 4 days. All rats were killed on the eighth day. Six control rats also were adrenalectomized and received 0.9% saline injections, the corticosterone vehicle. Routine blood counts were made before beginning injections, one day following adrenalectomy and at the termination of the experiment. Rats anesthetized with ether were exsanguinated by heart puncture and killed by decapitation. The femurs were cleaned of surrounding tissues and the bone mass determined before and after demedullation. This yielded the weight of the total femoral marrow. For this procedure, the femur was split along its main axis. After a sample of bone marrow was taken, the remainder was removed by swabbing the bone cavity clean with lens paper. The rat is well suited for these procedures because the shaft is occupied by red marrow and is free of spicules. A sample of bone marrow was drawn into a pipet calibrated to contain exactly 20 mm^3. The mass of this marrow was determined by weighing the pipet before and after taking the marrow sample. The bone marrow was then expelled into a test tube containing 2 ml homologous serum. A suspension was formed by drawing the marrow up and down in a rubber-nippled glass pipet. The preparation was also shaken for 5 minutes to insure a uniform cellular distribution. Samples were taken for hemacytometer counts and smears. One sample was diluted with Tyrode's fluid and another with Randolph's phloxine-methylene blue mixture(11). Having ascertained the total femoral mass, the first count provides data for determination of the total cellular numbers. With the aid of the second count, the total numbers of nucleated cells and eosinophils are determined. The difference between the two counts is the number of non-nucleated erythrocytes in the sample. Furthermore, using myelogram percentages obtained from stained smears, the absolute numbers of other cell types can be computed. Nucleated erythroid cells were identified from bone marrow smears treated by Ralph's technic(12) and counterstained with Harris' hematoxylin. Wright-stained smears were also examined.

Results. Table I indicates that corticosterone increased markedly the numbers of nucleated red blood cells in the femurs of adrenalectomized rats while the numbers of neutrophils and eosinophils decreased. In this series of animals the decreases in lympho-

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* Post-doctoral Damon Runyon Research Fellow.

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TABLE I. Effects of Corticosterone upon Bone Marrow Cells and Organ Weights of Adrenalectomized Rats (Means ± Standard Errors).

	Corticosterone	Saline	P†
Bone marrow wt (mg/f*)	78.4± 3.3	84.7± 5.8	.37
Nucleated red blood cells/f (millions)	63.8± 9.4	29.4± 4.5	.002
Non-nucleated red blood cells /f (millions)	118.1±21.6	96.2± 7.3	.35
Lymphocytes/f (millions)	19.6± 8.0	37.6± 8.1	.15
Neutrophils/f (millions)	48.4± 4.9	81.5± 8.0	.001
Eosinophils/f (millions)	6.1± 0.5	12.6± 2.6	.03
Misc. cells/f (millions)	23.4± 4.4	44.2± 11.6	.15
Thymus wt, mg	146 ±20	309 ± 32	.002
Spleen ” ”	884 ±63	1268 ±118	.02
Kidney ” ”	924 ±19	906 ± 28	>.56

* f = femur.

† P is calculated from the distribution of Fisher's t.

Values less than .05 have been considered significant.

cytes and miscellaneous cells (including damaged cells) were not statistically significant. Thymus weight was depressed sharply and spleen weight also underwent diminution following corticosterone treatment. Kidney weights were not affected. The steroid-treated rats lost about 5% of their initial body weights.

The corticosterone treated rats revealed a marked peripheral blood eosinopenia accompanied by a moderate neutrophilia. Although no lymphopenia was observed, the lymphocytosis evident in the saline-injected controls was not seen in the steroid-treated rats. Corticosterone treatment resulted in a slight, but significant, reticulocytosis. However, it exerted no discernible influence upon peripheral red blood cell counts, hemoglobin concentrations or hematocrit percentages.

Discussion. The present experiments confirm and extend the work of others(9,13-19) indicating erythropoietic stimulation following injections of ACTH or various adrenal steroids. It is interesting that such a strong bone marrow response occurs at a time when the only peripheral indication of erythroid stimulation is a slight reticulocytosis. It is felt that in order to evaluate the bone marrow response properly, the quantitative approach is a necessity. Myelogram percentages alone may accentuate unimportant relationships and yield no information concerning the actual numbers of cells present. This work represents an initial attempt to apply to bone marrow a parallel of that contributed to peripheral blood studies by blood volume determinations.

Summary. A method has been described for the determination of the total numbers of bone marrow cells in the rat femur. Administration of corticosterone to adrenalectomized rats results in significant increases in the numbers of nucleated erythroid cells and decreases in the numbers of neutrophils and eosinophils within femoral marrow.

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Relation of Lipolysis to Deturbidification and Returbidification in Heparin-Induced Lipemia-Clearing Phenomenon.* (21514)

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It has been shown that during *in vitro* clearing by post-heparin plasma free fatty acid is released(1,2,3). In this laboratory (4) it has also been established that an increase in free fatty acid concentration in the plasma occurs after injection of heparin, especially in the presence of lipemia. Whether lipolysis, with free fatty acid release, is an essential and primary part of the clearing phenomenon or whether it is a secondary associated effect has not been decided(3,5).

In this study the time course of deturbidification of lipemic serum by post-heparin plasma and by pancreatic lipase has been correlated with release of free fatty acid. The results are consonant with the hypothesis that lipolysis is the basic mechanism of the clearing phenomenon. The returbidification which occurs following *in vitro* clearing has also been studied and the findings support the hypothesis that it is caused by calcium soap formation which occurs when the quantity of free fatty acid released exceeds the capacity of the serum albumin to bind it.

Methods. Blood samples were obtained from male albino rats under ether anesthesia by aortic puncture. The blood was drawn into chilled syringes and maintained at 5°C or lower during centrifugation and storage prior to use. Control blood was taken from rats fasted for 16 hours. Lipemic blood was obtained from animals which had been given 2 ml of corn oil 3 hours previously. Post-heparin blood was obtained from rats given 10 mg/kg of heparin intravenously 10 minutes previously. Pancreatic lipase was prepared by extracting desiccated, defatted pancreas powder (Viokase, VioBin Corp.) with 0.05 M sodium diethyl barbiturate (2.5 mg/ml) and filtering. The coconut oil emulsion was a commercial preparation (Ediol, Schenley Laboratories) and was diluted to the desired concentration with distilled water just prior to use. *Optical density* was read against a distilled water blank at 700 mμ in a Coleman Junior spectrophotometer using microcuvettes. Free fatty acid was determined by the following method. One ml of sample was added with mixing to 1 ml of 0.2 M phosphate buffer pH 5.0 and 1 ml of distilled water in a glass stoppered test tube. Three ml of 95% ethyl alcohol was added and mixed. Three ml of petroleum ether (BP 30° to 60°C) was added and the tubes were shaken for 1 minute and then centrifuged in a refrigerated centrifuge for 2 minutes. The petroleum ether layer was removed with a syringe and needle and transferred to a 17 x 120 mm test tube. The extraction with petroleum ether was repeated two more times and the extracts combined. The petroleum ether was evaporated by immersion of tubes in a water bath which was gradually raised to 70°C until they were just dry. Two ml of 95% ethyl alcohol was added, the tubes were heated in a water bath at 90°C for 1 minute and titrated while hot with 0.02 N aqueous NaOH, using thymol blue as an indicator. A Rehberg microburet of 0.2 ml capacity was used for the titration and CO_2-free air was bubbled through the samples during titration. Free fatty acid determinations were done in duplicate. A water blank was run with each set of determinations. The *in vitro* systems studied had the following composition: A. 1 vol. post-heparin plasma, 1 vol. control serum, 2 vol. lipemic serum. B. 3 vol. pancreatic powder extract, 7 vol. lipemic serum. C. 10 vol. post-heparin plasma, 1 vol. 1% coconut oil emulsion. D. 2 vol. pancreatic powder extract, 8 vol. control serum, 1 vol. 1% coconut oil emulsion. E. 2 vol. pancreatic powder extract, 8 vol. 0.05 M sodium diethyl barbiturate, 1 vol. 1% coconut oil emulsion. The ingredients were brought to bath temperature (25°C for A, B,

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* The opinions expressed in this paper are those of the authors and do not necessarily represent those of any governmental agency.

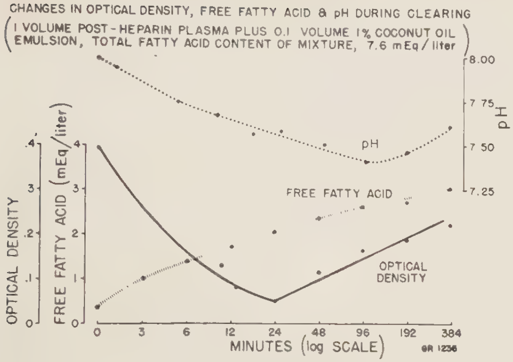

FIG. 1. Deturbidification and returbidification of coconut oil emulsion by post-heparin plasma. Release of free fatty acid proceeds during both phases.

C, and D, 37°C for E) and then mixed. Optical density readings and samplings for free fatty acid determination were made immediately after mixing and at intervals thereafter.

Results. Fig. 1 presents the results of an *in vitro* experiment (25°C) on the interaction of post-heparin plasma and coconut oil emulsion. The turbidity of the mixture decreased rapidly, reaching a value comparable to that of nonlipemic plasma within 24 minutes and then gradually rose again over a period of several hours. The free fatty acid concentration rose during the period of deturbidification and continued to rise while returbidification was occurring. The total fatty concentration remained constant, and by the end of 6 hours about 35% of the fatty acid was in the free form.

Experiments on deturbidification. In experiments in which pancreatic lipase was added to lipemic serum, deturbidification accompanied by a progressive rise in free fatty acid concentration was observed, comparable to the changes seen with post-heparin plasma. In Fig. 2 the results of a series of experiments with pancreatic lipase and post-heparin plasma are presented, plotting increment in free fatty acid against decrement in optical density. It will be noted that in the initial stages there is an essentially linear relation between deturbidification and free fatty acid release. In the case of the post-heparin plasma system this is followed by a phase in which deturbidification becomes much slower or stops while release of free fatty acid continues. Although there is considerable variation in the absolute position of the curves, the slope of the initial portion is similar in all instances and is approximately the same for pancreatic lipase and post-heparin plasma. Control studies with lipemic plasma alone showed only small changes in both free fatty acid and optical density, even when the period of observation was greatly extended.

A similar series of experiments was conducted in which coconut oil emulsion was used in place of lipemic serum. The results with the two substrates were quite similar. Once again, during the initial phase of the reaction a linear relation between deturbidification and free fatty acid release was seen. Furthermore, in an experiment in which serum was omitted deturbidification accompanied lipolysis of coconut oil emulsion by pancreatic lipase. A higher temperature was required to produce the reaction and the final extent of deturbidification was less in the absence than in the presence of serum. Sodium desoxycholate (10 mg/ml) completely inhibited deturbidification and reduced free fatty acid release to very low levels. Eserine sulfate (0.01 mg/ml) had no effect on either deturbidification or free fatty acid release.

Experiments on Returbidification. Fig. 3 presents the results of an experiment on the effect of oxalation of post-heparin plasma on deturbidification and returbidification with coconut oil emulsion substrate. Deturbidifi-

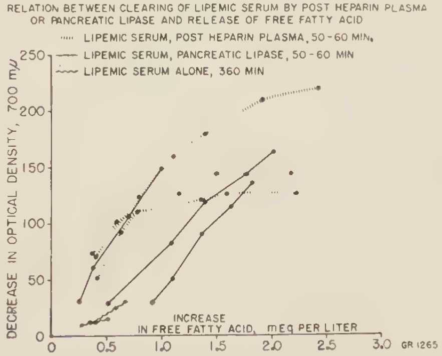

FIG. 2. Each line represents a separate experiment during which samples were taken at intervals for optical density and free fatty acid measurements. The times given on the graph are the times of the last sample. Decreases in optical density are expressed in optical density units multiplied by 1000.

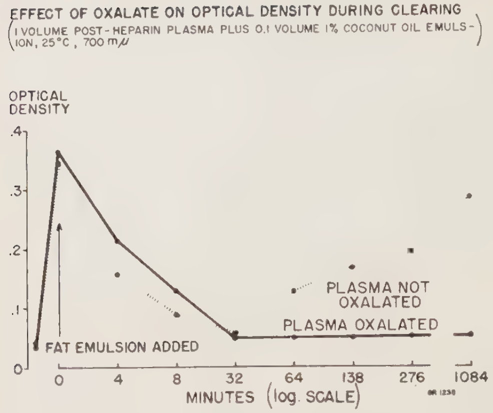

FIG. 3. Failure of returbidification to occur in oxalated plasma.

cation was essentially unaltered by oxalation, whereas returbidification occurred only in the unoxalated system.

Fig 4 presents the results of studies on the turbidity produced by adding sodium oleate to serum or post-heparin plasma. Not until the concentration of oleate exceeds about 2 meq./l does the optical density rise above that of plasma or serum alone. Beyond this concentration the optical density is approximately linearly proportional to oleate concentration. In control studies, addition of sodium oleate to oxalated serum produced no change in optical density over this same range of concentrations of oleate.

Discussion. These experiments clearly demonstrate that the lipolytic activity of rat plasma is greatly enhanced by heparin injection using either a synthetic emulsion or lipemic serum as a substrate. The results further suggest that the clearing activity of post-heparin plasma is related to its enhanced lipolytic activity. This view is supported by these observations: a) that in the initial stages of deturbidification a linear relation exists between release of free fatty acid and decrease in optical density, b) that addition of pancreatic lipase to lipemic serum produces deturbidification with a relation between loss of turbidity and increment in free fatty acid comparable to that with post-heparin plasma and c) that sodium desoxycholate inhibits both clearing and lipolysis by post-heparin plasma. The last observation indicates that the lipase activity induced by heparin injection is different from pancreatic lipase, although both are capable of producing deturbidification. The lack of effect of eserine on lipolysis or clearing would indicate that the post-heparin lipase is not simple plasma pseudo-cholinesterase(7). Levy and Swank (5) found an elevation in tributyrin esterase in dog plasma following heparin injection. This suggests that the esterase activity of heparin-induced lipase extends to this substrate in this species.

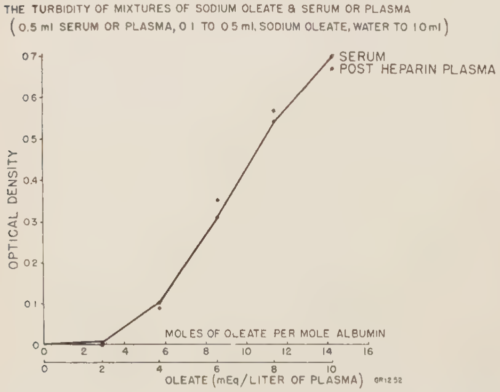

FIG. 4. Turbidity produced by adding increasing amounts of sodium oleate to serum from an untreated rat and to plasma from a rat which had received an injection of heparin.

The view that post-heparin clearing is essentially a lipolytic process is fully supported by the studies of Robinson and French(6) who used *in vitro* systems of post-heparin rat plasma and rat chyle and were able to recover all of the fat which left the turbid form as unesterified fatty acid bound to albumin.

In vitro clearing involves no change in the total amount of fatty acid present in the system. *In vivo* heparin-induced lipemia-clearing is attended by a precipitous drop in total fatty acid level in the plasma(4). Presumably, the fat which has been transformed to a non-turbid form leaves the blood very quickly. The findings of Robinson and French(6) cited above suggest that this rapidly removed fat is transported from blood to tissues as a complex of albumin and free fatty acid.

The concentration of coconut oil fatty acid in the incubation mixtures was 3.9 meq./l. The release of free fatty acid never equaled this theoretical amount even when clearing was essentially complete, *i.e.*, when the optical density of the system was lowered to the value of non-turbid plasma (about 0.05). The ratio of percent change in optical density to percent of coconut oil total fatty acid released in free form was about 1.4, indicating that complete hydrolysis of the triglyceride of the turbid emulsion is not required to render it non-turbid. Furthermore, it cannot be assumed that all of the free fatty acid which appeared in the incubation mixture arose from the hydrolysis of turbid triglyceride because release of free fatty acid continues after turbidity has been abolished (Fig. 1), and free fatty acid is released when non-turbid post-heparin plasma is incubated without added turbid substrate(4). Thus the conclusion seems warranted that the lipolytic action of post-heparin plasma can affect non-turbid as well as turbid substrates.

The present studies on returbidification are consonant with the view of Nichols *et al.*(1) that this is due to formation of calcium soaps. The prevention of returbidification by decalcification of plasma with oxalate and the production of comparable turbidity by the addition of amounts of fatty acid comparable to that which is released during clearing supports this view. The latency of returbidification can be ascribed to the fact that calcium soaps do not form until the concentration of free fatty acid is in excess of that which can be bound by albumin.

Summary and conclusions. 1. During the clearing of lipemic serum or coconut oil emulsion by postheparin plasma *in vitro* release of free fatty acid occurs. During the initial portion of the reaction there is an essentially linear relation between the release of free fatty acid and the decrease in turbidity. Later, release of free fatty acid continues but decrease in turbidity stops or returbidification occurs. Pancreatic lipase may be substituted for post-heparin plasma to produce clearing of lipemic serum or coconut oil emulsion and a similar relation between lipolysis and turbidity is found in these systems. The increased lipolytic activity of post-heparin plasma differs from pancreatic lipase in that it is inhibited rather than activated by sodium desoxycholate. The returbidification which sometimes follows clearing is probably due to calcium soap formation because it can be prevented by removal of calcium with oxalate and it can be simulated by addition of sodium soap to plasma. 2. It is concluded that the *in vitro* lipemia clearing activity of post-heparin plasma can be quantitatively accounted for on the basis of its lipolytic activity.

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New Procedure for Ascorbic Acid Analysis by the Osazone Method.* (21515)

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Roe and Keuther(1) described a method for determining ascorbic acid in which the vitamin is oxidized to dehydroascorbic acid by Norit. In the presence of thiourea the osazone is formed by incubation with 2,4-dinitrophenyl-hydrazine. After the addition of 85% sulfuric acid, the red-colored solution is read at 540 mμ in a spectrophotometer. Roe and Oesterling(2) utilized the osazone method to determine ascorbic acid and oxidized ascorbic acid simultaneously by omitting the preliminary oxidation step in one aliquot of the sample. Bolomey and Kemmerer(3) modified this method by substituting glacial acetic acid for 85% sulfuric acid. This avoided the time-consuming, dropwise addition of sulfuric acid but led to a pronounced decrease in sensitivity. Bolin and Book(4) further modified the method by replacing Norit with 2,6-dichlorophenolindophenol (DCPP), which considerably facilitated the oxidation of ascorbic acid. The last 2 modifications have been applied in combination by Williams(5), who found that the resulting procedure gave values in fair agreement with the indophenol titration method. However, serious defects in the combined procedure were observed: (1) poor sensitivity when glacial acetic acid was used for color development and (2) a source of error inherent in the Bolin and Book modification because of significant light absorption by reduced DCPP at 540 mμ (5 galvanometer units/drop of 200 mg% DCPP, with the Evelyn colorimeter). In the Bolin and Book procedure, the dye is omitted from the sample blanks and from the tubes in which dehydroascorbic acid is measured. Thus a significant error can arise since the dye is added to all other tubes.

Methods. To increase the sensitivity of the Bolomey and Kemmerer modification(3), we investigated the use of acids other than glacial acetic acid or 85% sulfuric acid. Of the different acids and combinations of acids tested, the best results were obtained with a 2:3 mixture of 85% phosphoric and concentrated hydrochloric acids. As shown in Fig. 1, this mixture gave a 2-fold increase in color development over that observed when glacial acetic acid was used.

To overcome the error introduced by omitting DCPP from certain tubes, the dye was added in the same amount to all the tubes, but in such a manner that both ascorbic acid and dehydroascorbic acid could be measured separately. The dye was added to the thiourea solution *before* the sample was added to the tube in which dehydroascorbic acid was to be measured. Thus the dye was reduced by the thiourea before the sample was added, pre-

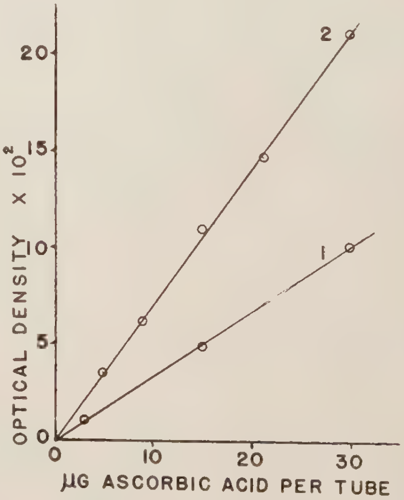

FIG. 1. Standard curves obtained in the osazone method of ascorbic acid analysis. Curve 1—Glacial acetic acid used for color development. Curve 2—85% phosphoric and concentrated hydrochloric acids (2:3 mixture) used for color development. All tubes read at 540 mμ.

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TABLE I. Recovery of Added Ascorbic Acid from Samples of Rat Liver Extract.

Sample	Ascorbic acid in µg/tube Found	Calculated
1 ml of rat liver extract*	15.5	
.5 " *Idem*	7.9	7.8
.5 " " + 9 µg asc. acid†	16.5	16.9
.5 " " + 15 µg asc. acid	22.9	22.9

* See text for preparation.
† Ascorbic acid.

venting oxidation of the ascorbic acid by the dye but still allowing measurement of dehydroascorbic acid in the presence of the dye.

The validity of these modifications was checked by ascorbic acid recovery experiments and by studying the possible interference of other substances which might form measurable osazones under the conditions of the analysis. The recoveries of known amounts of ascorbic acid from an extract of rat liver, obtained by homogenizing rat liver in 5% metaphosphoric acid – 10% acetic acid and centrifuging, are presented in Table I. The recovery of ascorbic acid was very close in all cases to the theoretical values. When interference by glucose, fructose, and glucuronolactone was studied, it was found that levels of glucose and fructose as high as 1500 µg gave no significant absorption (galvanometer readings of 99 and 98, respectively, as compared to 100 for the reagent blank). Glucuronolactone at a 50 µg level also did not interfere. At a level of 1500 µg, it gave a turbid solution. However, since 5 µg of ascorbic acid can be easily determined by this method and 50 µg of glucuronolactone cause no interference, the ratio of glucuronolactone to ascorbic acid must be greater than 10:1 before interference from this compound need be considered. Probably in few natural substances to be assayed for ascorbic acid would this ratio be approached.

Reagents. Ascorbic acid (Merck). A solution of 1.5 mg per ml in a mixture of 5% metaphosphoric acid in 10% acetic acid is stable for several months at –5°C. *5% Metaphosphoric acid in 10% acetic acid.* This is stable for at least a week at room temperature and is used for extraction and dilution of samples. *2,6-dichlorophenolindophenol.* 100 mg of dye are dissolved in 50 ml of water and the solution is filtered. This solution can be stored at 5°C for a month. *Thiourea solution.* One per cent thiourea in 5% metaphosphoric acid. If turbid, it should be filtered. *2,4-dinitrophenylhydrazine solution.* A 2% solution is prepared in a solution of 1 part concentrated sulfuric acid and 3 parts of water. This is stored at –5°C and is filtered before use. *A solution of 3 parts of concentrated hydrochloric acid and 2 parts of 85% phosphoric acid.* This reagent should be prepared just before use. Procedure. A series of standards are prepared in 5% metaphosphoric acid – 10% acetic acid ranging from 0 (reagent blank) to 30 µg ascorbic acid per 2 ml. When natural materials are assayed for ascorbic acid, the materials are deproteinized and diluted with the solution of 5% metaphosphoric acid in 10% acetic acid so that the ascorbic acid concentration is 5-10 µg/ml. Two ml of the samples are taken for analysis. Three test tubes are required for the simultaneous analysis of total ascorbic acid and dehydroascorbic acid in 1 sample. One drop of DCPP solution and 2 ml of thiourea solution are mixed in tube 1. Two ml of sample are added to this tube and to tubes 2 and 3. One drop of dye is then added to tubes 2 and 3 and to the standards, followed by the addition of 2 ml of thiourea solution. One ml of the 2,4-dinitrophenylhydrazine solution is then added to the standards and to tubes 1 and 2. The contents of the tubes are mixed well and are incubated at 37°C for 3 hours. After incubation, 5 ml of the hydrochloric acid-phosphoric acid solution are added to all tubes. One ml of the 2,4-dinitrophenylhydrazine solution is then added to tube 3. All tubes, after mixing, are read at 540 mµ in an Evelyn Colorimeter. Tube 1 gives the amount of dehydroascorbic acid present; tube 2, the total ascorbic acid present; and tube 3 is the sample blank.

Summary. The osazone method for ascorbic analysis(1) and a number of published modifications of this method(2-4) have been evaluated to derive a procedure which is simultaneously sensitive, specific, accurate, and easy to carry out for routine analyses. In the resulting method, the time-consuming use

of Norit and 85% sulfuric acid(1) is avoided, the sensitivity is twice that obtained when glacial acetic acid(3) is used, and a source of error introduced with the use of 2,6-dichlorophenolindophenol(4) to oxidize ascorbic acid before formation of the osazone is eliminated. The resulting method has been found to be quite satisfactory with respect to sensitivity, specificity, facility, and recovery of added ascorbic acid.

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Effect of Antibacterial Agents on Ammonia Production within the Intestine.* (21516)

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The gastrointestinal tract is a major source of the ammonia found in the peripheral blood and, as demonstrated by Folin and Denis(1, 2), more ammonia is formed in the colon than in any other part of the alimentary canal. The dense bacterial population of the colon is undoubtedly responsible for this fact since the ammonia is produced within the intestine as a consequence of bacterial action on nitrogenous substrates. In an effort to diminish the production of ammonia within the intestine and thus reduce the quantity delivered to the peripheral blood, orally administered antibacterial agents have been used. McDermott and coworkers(3,4) and others(5,6) have used these drugs together with other therapeutic measures in human patients. Although it is difficult to differentiate the effect of the antibiotics from that of the other measures which were concomitantly employed, they have reported that the patients were benefited. Other workers (7,8) have been unable to demonstrate a significant reduction of the ammonia content of the peripheral blood in experimental animals treated with antibiotics. However, in these experiments, the initial values for ammonia in the peripheral blood were too low adequately to reflect any change which might have been induced by the antibiotics.

This study is concerned with the origin of the ammonia in the blood and with the changes in the ammonia content of portal blood produced by certain antibacterial substances.

Methods. Each of 5 adult mongrel dogs was fed approximately 750 g of raw horsemeat daily for 3 days. On the fourth day, an exploratory laparotomy was performed using intravenous sodium nembutal anesthesia and strict aseptic precautions. Samples of blood were taken from the venous return of (1) the descending colon, (2) the mid small intestine, or (3) the stomach, and a fourth sample was taken from the inferior vena cava. Heparin was used as an anticoagulant. The ammonia content of each sample of blood was determined at once by the microdiffusion method of Conway(9). A sample of feces was then removed for culture through a small colotomy made in the descending colon adjacent to the area from which the first blood sample was obtained. To study the effects of antibacterial agents, the procedures described above, including the feeding of horsemeat, were repeated on the same animals after an interval of 2 to 3 weeks. The samples of blood and the cultures of feces were taken from areas as close as possible to those used for the previous op-

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* Supported by the Christine Breon Fund for Medical Research.

TABLE I. Results of Determinations in 9 Experiments on Ammonia Content of Venous Blood from Various Segments of Gastrointestinal Tract and from a Peripheral Vein before and after Treatment with Antibacterial Agents.

	Blood ammonia nitrogen (μg/ml)			
Source of blood	Control	Neomycin	Sulfasuxidine	Achromycin
Dog 62				
Colon	22.02	2.09	15.1	12.19
Small intestine	1.64	1.63	.99	1.97
Stomach	1.09	.74	2.43	.38
Inferior vena cava	.95	.06	1.01	.69
Dog 63				
Colon	13.77	3.73	21.87	9.61
Small intestine	1.81	.83	2.90	1.27
Stomach	.34	.76	.78	.50
Inferior vena cava	.44	.14	2.90	.21
Dog 154				
Colon	13.59	.56		
Small intestine	1.44	.97		
Stomach	1.78	.50		
Inferior vena cava	1.08	.66		
Dog 160				
Colon	16.49	8.42		
Small intestine	2.76	2.95		
Stomach	.08	.45		
Inferior vena cava	.48	.42		
Dog 190				
Colon	6.96	2.53		
Small intestine	1.78	4.02		
Stomach	.19	.45		
Inferior vena cava	1.06	.67		

erations. Five animals were given orally 1 g of neomycin every hour for 4 hours, followed by one gram every 4 hours for 8 hours on the day prior to laparotomy. Two animals were used to study the effects of sulfasuxidine and achromycin on ammonia production within the intestine. During one test period sulfasuxidine was administered orally in doses of 5 grams 3 times daily for 5 days prior to operation, and in a second test period achromycin, 2 grams daily by mouth for 3 days prior to operation. In 3 animals used for testing the effect of neomycin on the production of ammonia within the intestine, the fecal samples obtained by colotomy were plated out on differential media and bacterial colony counts of the coliform group, enterococci, and clostridia were made according to the method of Lockwood *et al.*(10).

Results. The ammonia content of the blood taken from the veins of various segments of the gastrointestinal tract and from a peripheral vein, before and after treatment with antibacterial substances, is shown in Table I. A typical response in one animal is shown graphically in Fig. 1. It is apparent that the colon contributes significantly larger amounts of ammonia to the portal venous blood than any other part of the gastrointestinal tract which was tested. For example, in one animal (No. 62), the level of ammonia in the venous return from the colon was as high as thirteen times that found in the venous blood returning from the other regions of the gastrointestinal tract. These high levels of ammonia in the colonic venous (portal) blood facilitated the detection of any effect which antibacterial agents might exert on the production of ammonia within the intestine.

The ammonia levels in the blood returning from the stomach and small intestine are similar to those found in the peripheral blood. The contrast between colonic venous blood levels of ammonia and those of the peripheral blood is striking. This emphasizes the role that the liver normally plays in detoxifying the large amounts of ammonia brought to it by the portal blood. If ammonia were to be present in the peripheral blood in similar quantities, grave central nervous system symptoms would be likely to occur.

Of the antibacterial agents tested, neomycin was the most effective in reducing the ammonia level of the venous blood from the colon (Table I). In all animals, the ammonia content of these samples of venous blood from

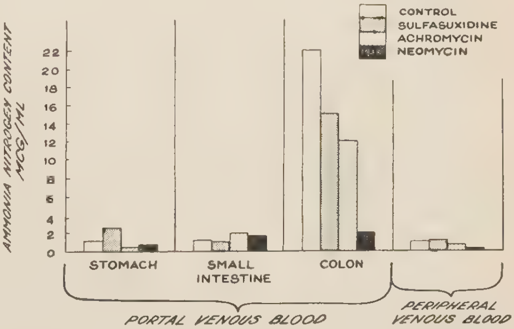

FIG. 1. Ammonia content of blood from various segments of gastrointestinal tract and from a peripheral vein before and after treatment with antibiotics or sulfasuxidine.

the colon was decreased to a value which was less than half that of the original control value, and in 2 animals, the reduction was more than tenfold. With neomycin the ammonia levels in the peripheral blood were also decreased in every animal, but these changes were too slight to be of noteworthy significance in contrast to the highly significant alterations found in the portal venous blood samples from the colon.

In studies on effect of sulfasuxidine or of achromycin on ammonia content of blood in various portal radicals, it was found that neither substance produced significant changes in ammonia levels of any of the blood samples tested.

After treatment with neomycin, a reduction in the total bacterial content of the colon was observed, but the greatest decrease occurred in the numbers of coliform organisms.

Summary. 1. Five mongrel dogs were fed raw horsemeat 3 days and on fourth day, a laparotomy was performed. Samples of venous blood from various segments of gastrointestinal tract were drawn for determinations of ammonia content. The same animals, similarly prepared, were used after an interval of 2 to 3 weeks to test the effect of orally administered antibacterial agents on the level of ammonia in venous blood obtained from various portions of the gastrointestinal tract. For this purpose, neomycin, sulfasuxidine or achromycin were administered preoperatively by mouth for varying periods. 2. The colon was the principal source of ammonia in portal blood. Of three antibacterial substances tested, only neomycin markedly reduced the ammonia content of portal venous blood from the colon.

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Immunity in Group A Streptococcal Infections I. Non-Specific Protective Effect in Repeated Pulmonary Infections.* (21517)

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(Introduced by C. G. Harford.)

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The concept that antibacterial immunity in Group A streptococcal infections is type specific is supported both directly and indirectly by considerable evidence(1-5). Conversely, however, a number of published studies suggests that a measure of group-specific immunity may also exist(6). In the course of developing methods for production of streptococcal pneumonia in rats, certain observations relating to the latter type of immunity were made, and form the basis of this report.

Methods. Induction of streptococcal pneumonia. Technics employed for induction of streptococcal pneumonia have been described

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* Supported by grant from Life Insurance Medical Research Fund.

TABLE I. Penicillin Dosage Schedule Used in Treatment of Streptococcal Pneumonia.

Hr after infection	Penicillin (units)
18	6000
24	6000
42	3000
66	3000
90	3000
114	3000
138	3000
162	3000

in detail(7). To characterize the nature of repeated infections, animals were infected with a given strain and 18 hours after infection were treated with aqueous procaine penicillin‡ by intramuscular injection according to dosage schedule shown in Table I. One week after completion of therapy reinfection was attempted by introducing a serologically unrelated group A strain into the lung opposite that originally infected. Rats were sacrificed 24 hours after second infection was induced, and sections for microscopic study prepared from the more recently infected lungs according to technics previously outlined(7). The first inocula were introduced into the lower lobe bronchus of left lung and the second ones into the lower or cardiac lobes of right lung. *Cultures.* Strains IRS-100 (type 26) and 1GL-23 (type 17) were originally supplied by Dr. Maclyn McCarty and strain C-203 (type 14) by Dr. Rebecca Lancefield of the Rockefeller Institute for Medical Research. Strain 41439 (type 3) was received from Dr. Alton J. Morris of the Streptococcal Disease Laboratory, Ft. Warren, Wyoming. Cultures were grown and the bacteria-mucin suspensions prepared as described earlier(7). In subsequent experiments the mortality rate for a second infection was determined for groups of rats previously infected and compared with the mortality rate resulting (a) in normal rats infected for the first time; and (b) in animals which had been inoculated with sterile gastric mucin and treated with penicillin according to the schedule shown in Table I.

Results. Histologic observations. Microscopic sections made from lungs which were the sites of second infections were compared with sections obtained from animals undergoing first infections. Whereas in the latter the characteristic findings were those of a spreading lesion with many free streptococci in edema fluid (Fig. 1), in the sections from reinfected animals organisms were rare (Fig. 2) and the cellular response less intense; proportionately fewer polymorphonuclear leucocytes were seen, and when streptococci could be found, they were noted within phagocytes.

These initial observations suggested the possibility that first infections conferred nonspecific protection to some degree against a second infection, but it was necessary to exclude the presence of leucocytes in the right lung prior to the second infection, since the importance of these cells in the host defense against acute bacterial infections has been amply demonstrated(7,8). In streptococcal pulmonary infections some spread of bacteria and cells occurs via the bronchi(7), and it was, therefore, conceivable that leucocytes might have reached the right lung at the time of the original infection and remained within the alveoli for a period of several weeks. In order to determine the effect, if any, of an initial left-sided infection on the contralateral lung, the following experiment was done. Six rats were infected on the left side and treated with penicillin for seven days. One week after conclusion of treatment, the animals were sacrificed and sections were made from the right lungs. Careful microscopic studies revealed no structural deviation from the normal, and no cells were seen within the alveoli.

These observations indicated that the first infection did not lead to structural changes in the opposite lung nor to an increased number of phagocytic cells within the alveoli.

Mortality Studies. To compare the mortality rate for reinfection with that of primary infection, the following experiments were performed. Rats were infected on the left side, treated with penicillin and one week after completion of therapy were reinfected with a different type of streptococcus. The animals were observed for 14 days after the second infection.

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‡ Aquacillin® Schenley, kindly supplied by Mr. C. B. Taft of Schenley Laboratories, New York City.

FIG. 1 and 2. Microscopic sections from lungs of rats stained by Gram-Weigert technic. Photomicrographs taken by Mr. K. Cramer Lewis, Department of Illustration, Washington University School of Medicine.

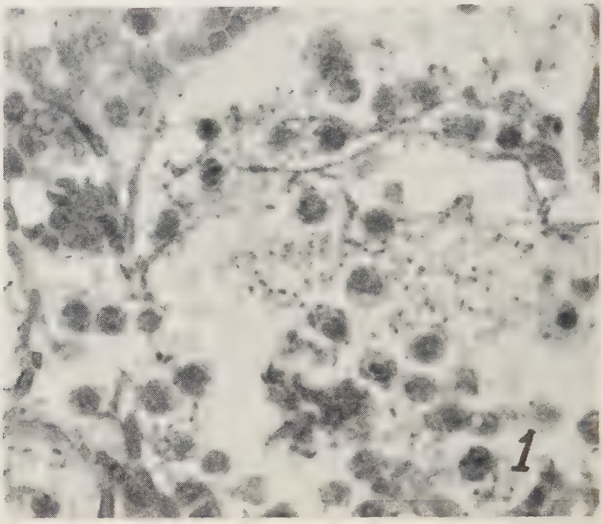

FIG. 1. First infection lesion, 24 hr after induction, showing edema fluid, polymorphonuclear exudate and free streptococci. ×600.

Table II shows the results of 3 such experiments done at different times and with different combinations of strains, and Table III presents an overall summary. It should be noted that in all experiments, the challenging infection was induced in both previously infected and control groups at the same time under identical circumstances. These results, when subjected to statistical analysis, were found to be significant, and, therefore, constitute further evidence that some degree of protection against subsequent streptococcal pulmonary infection is induced, under the conditions of the experiment, by a prior pneumonic infection. That the protective effect is not solid is suggested by the third experiment in Table II. Additional experiments of the same type have confirmed the fact that the protective effect is limited and can be easily obscured unless the virulence of the challenging infection is set within narrow limits.

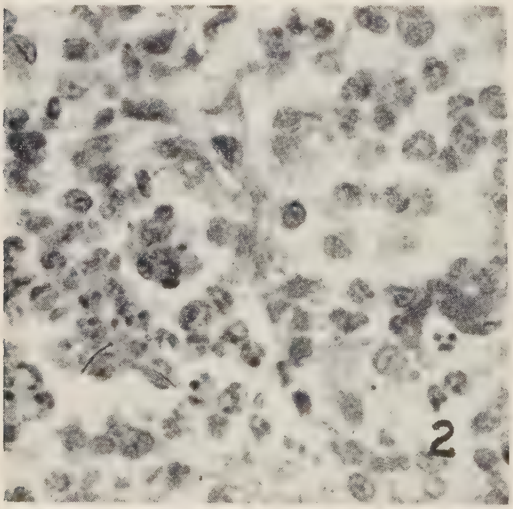

FIG. 2. Second infection lesion 24 hr after induction. There is no edema response and no free streptococci are seen. ×600.

The use of repository penicillin raised the question as to whether persistence of the antibiotic in the treated animals could explain their relative resistance to the second infection. As one means of excluding this possibility, the following study was done. Fourteen rats were infected with IGL-23 and treated with penicillin as before. Five rats received an inoculum which was identical except that it contained no streptococci. This group of animals was also treated with penicillin. One week after completion of the penicillin course, both groups of treated animals and a third consisting of thirteen normal rats were infected with strain 41439. The results of this experiment are presented in Table IV.

TABLE II. Mortality of First and Second Infections with Streptococcal Pneumonia.

Animal group	Strain employed		No. of animals	Survived	Died
	1st infection	2nd infection		%	%
Reinfection	IRS-100	IGL- 23	21	19 (90)	2 (10)
Control	—	”	22	4 (18)	18 (82)
Reinfection	IGL- 23	IRS-100	10	9 (90)	1 (10)
Control	—	”	7	2 (28)	5 (72)
Reinfection	IGL- 23	C-203	12	4 (33)	18 (67)
Control	—	”	9	0 (0)	9 (100)

TABLE III. Total Mortality of First and Second Infections with Streptococcal Pneumonia.

Animal group	No. of animals	Survived	Died
Reinfection	43	32 (74)	11 (26)
Control	38	6 (16)	32 (84)

These findings are of interest in two regards. Whereas the mortality rate for the reinfected animals was 54%, that for the mucin-penicillin animals was 20%; although the series is too small for statistical analysis, a distinct difference in the outcome was apparent. Further, the normal controls succumbed in significantly greater numbers than did the reinfected animals ($\chi^2 = 1.4$), again substantiating the observations shown in Tables II and III.

Although the available data indicate that penicillin, when administered parenterally as an aqueous procaine preparation, cannot be detected in the serum for more than 72 hours at most(9-11), it was deemed advisable to determine whether levels of drug might persist in lung tissue, and thus explain the protective effect. The following study was, therefore, done. Three normal rats were treated with procaine penicillin according to the same schedule used for the other experiments. Seven days after completion of this regimen, these animals and 3 untreated control rats were sacrificed, and the left lung and cardiac lobe of the right lung were removed from each rat aseptically. Each portion of lung was thoroughly ground separately in a mortar with sand, and then taken up in 3.0 ml of tryptose phosphate broth. The gross sediment was removed by centrifugation and discarded. One ml of each tissue suspension was added to 9.0 ml of tryptose phosphate broth and 2-fold dilutions made. Tubes of tryptose phosphate broth containing decreasing concentrations of crystalline penicillin (0.5 μ/ml - 0.03 μ/ml) were prepared concomitantly. Each of the tubes was then inoculated with 0.1 ml of a rapidly-growing (4-hour) subculture of strain IGL-23. Gross observations of the rate of growth on the basis of turbidity were made at two and four hours, and blood agar plates were poured at 4 hours using 1.0 ml aliquots from each tube. Although the strain of streptococcus employed was exquisitely sensitive to penicillin (inhibited by a concentration of 0.03 μ/ml), no gross difference in the rate of growth was noted in the tubes containing lung suspensions from the treated or control animals, and growth on the poured plates made from control and penicillin-treated lung suspensions was identical; the poured plates made from the penicillin control cultures showed no colonies. These results demonstrated, therefore, that retention of penicillin in the tissues *per se* did not explain the protective effect.

Discussion. The observations presented herewith suggest that, under the conditions

TABLE IV.

Animal group	Inoculum		No. of animals	Survived	Died
	1st infection	2nd infection			
Reinfection	IGL-23	41439	13	7 (54)	6 (46)
Treated control	Sterile mucin	”	5	1 (20)	4 (80)
Untreated ”	—	”	13	3 (23)	10 (77)

employed, an initial streptococcal pneumonic infection confers some protective effect against a subsequent infection with a strain of different type, introduced into the opposite lung. The degree of protection is limited, and can easily be obscured if the virulence of the challenging infection is not controlled within narrow limits. The experiments described indicate clearly that the protective effect cannot be attributed to retention of the penicillin used to control the initial infection, but whether it is due to a humoral antibody or not is conjectural at this time. To date a systematic attempt to isolate and characterize such an antibody has been unrewarding(12), and the fact that early treatment of streptococcal infections tends to prevent or inhibit development of at least certain streptococcal antibodies(13-15) may be taken as some evidence against the explanation that the protection observed here is due to an antibody. On the other hand, the pattern by which streptococcal antibodies develop is not uniform(15), and as yet undefined antibodies may exist which are not inhibited by antimicrobial therapy. Since the host defense mechanism in the lung is a particularly effective one(8), it may be postulated that extremely small increments of protective antibody could shift the balance in favor of the host under the conditions of these experiments.

Despite the fact that the balance of evidence at hand speaks solely in favor of type-specific immunity, the scattered published results pointing to the existence of group-specific protection merit further investigation, especially since the ultimate control of streptococcal infections, if indeed it can be achieved, appears to depend on the development of some form of group-specific vaccine rather than on a polyvalent type-specific vaccine(16).

Studies at present under way in this laboratory are directed toward further exploration of the problem of group specific immunity in streptococcal infections, and will be reported later(12).

Summary. 1. It was noted that after pneumonic infection with group A streptococci and treatment with procaine penicillin, rats exhibited a degree of protection against a second pulmonary infection, induced in a different lobe with a strain of different type, not demonstrable in previously uninfected animals. This non-specific protective effect was of limited degree and could be obscured when the challenging infections were especially virulent. 2. Evidence is presented which indicates that the protective effect noted is not due (a) to the presence of phagocytes and/or structural alterations prior to the induction of the second infection, or (b) to the persistence of inhibitory levels of penicillin in the lung tissue *per se*. 3. The implications of these observations are discussed.

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Failure of Ethionine to Alter Gastric Secretion from Heidenhain Pouches in Animals. (21518)

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Ethionine, a synthetic amino acid which is the ethyl analogue of methionine, produces a variety of widespread effects in experimental animals including fatty infiltration of the liver(1) and destruction of the acinar part of the pancreas(2) with consequent impairment of pancreatic secretion(2,3).

The present experiments have been done to determine the effect of ethionine on secretion of pepsin and hydrochloric acid by canine gastric mucosa.

Methods. Heidenhain pouches drained by vitallium cannulas were made in 5 mongrel dogs. These animals, together with 2 intact dogs, were fed 200 g of a Cowgill synthetic diet(4) containing 30 g of casein (approximately 0.84 mg of methionine) for 6 days of each week. Gastric juice from the Heidenhain pouches was collected for periods of 24 hours. The volume of juice secreted during 24 hours after feeding was measured. The concentration of free hydrochloric acid was determined by titration against a 0.1 normal solution of sodium hydroxide with Töpfer's reagent as an indicator and is expressed in milliequivalents per liter. The daily output of hydrochloric acid was calculated. An aliquot of each sample was analyzed for pepsin activity by the method of Anson(5) in which hemoglobin serves as the substrate. One unit of pepsin is that amount which liberates 1 x 10^{-4} mEq. of tyrosine from 5 ml of the substrate in 10 minutes at 37°C. A series of such tests carried out before administration of ethionine was begun constituted control data to be compared with a similar series of tests done during daily oral administration of various doses of ethionine. The responses of the pouches to histamine given subcutaneously also were determined in 4 dogs before and at intervals during the time ethionine was being administered. Histamine was given every 15 minutes for 3 hours in doses of 0.1 mg for the first hour, 0.2 mg for the second and 0.4 mg for the third hour. The volume and free acidity of gastric juice collected every 15 minutes were measured. The maximal response of a pouch to histamine was determined by averaging the 3 highest values for volume and free hydrochloric acid secreted during 15-minute periods in each test. The ethionine was given orally in all instances. Dogs 1 and 2 received 1 and 2.5 mg per kilogram of body weight daily, respectively, for 9 weeks after which each was given 10 mg per kilogram of body weight daily for an additional 14 weeks. Doses of ethionine for dogs 3, 4 and 5 were 5, 10 and 25 mg per kilo of body weight per day respectively.

Results. The 2 dogs which were fed the

TABLE I. Mean 24-Hr Volume of Gastric Juice Secreted by Heidenhain Pouches in Dogs before and during Oral Administration of Ethionine.

		Vol of gastric juices				
		Before ethionine		During ethionine		
Dog	Daily dose of ethionine, mg/kg body wt	Collections	Mean vol, ml	Collections	Mean vol, ml	Mean diff., ml and stand. error
1	1	16	25	30	41	+16 ± 4.6*
	10	16	25	34	36	+11 ± 5.3*
2	2.5	16	43	31	53	+10 ± 6.1
	10	16	43	35	24	−19 ± 2.3*
3	5	13	27	13	34	+ 7 ± 3.7
4	10	12	69	39	55	−14 ± 7.3
5	25	15	28	7	25	− 3 ± 9.4

* Mean difference was significant at 5% level.

TABLE II. Mean Concentration and 24-Hr Output of Free Hydrochloric Acid from Heidenhain Pouches in Dogs before and during Oral Administration of Ethionine.

Dog	Daily dose of ethionine, mg/kg body wt	Free hydrochloric acid						Mean diff. and stand. error	
		Before ethionine			During ethionine				
		Collections	mEq./l	mEq./24 hr	Collections	mEq./l	mEq./24 hr	mEq./l	mEq./24 hr
1	1	16	22	0.5	30	68	3.0	+46 ± 9.9*	+2.5 ± 0.2 *
	10	16	22	0.5	34	18	0.8	− 4 ± 9.0	+0.3 ± 0.44
2	2.5	16	48	2.2	31	67	3.8	+19 ± 9.1*	+1.6 ± 0.78*
	10	16	48	2.2	35	43	1.0	− 5 ± 8.6	−1.2 ± 0.34*
3	5	13	68	2.1	14	81	2.7	+13 ±11.7	+0.6 ± 0.65
4	10	12	58	4.3	39	45	2.8	−13 ± 9.9	−1.5 ± 0.9
5	25	15	44	1.4	7	51	1.2	+ 7 ±12.5	−0.2 ± 0.9

* Mean difference was significant at 5% level.

diet without ethionine ate well and gained 1.5 and 2.5 kg in weight during a 4-month period, indicating that the diet was adequate.

The general effects of ethionine conformed with the pattern, previously reported from this laboratory, in experiments in which the amino acid was given orally in comparable doses to dogs(3). Dogs 1 and 2 did not show evidence of anorexia, loss of weight or jaundice, which are typical indications of ethionine intoxication in the dog, when they were receiving 1 and 2.5 mg/kg of body weight daily. Anorexia and loss of weight occurred when the daily dose was increased to 10 mg per kilo of body weight, and at necropsy fatty infiltration of the liver and atrophy of the pancreas were evident. Dog 3 had to be sacrificed after receiving ethionine in a dose of 5 mg per kilo of body weight daily for 6 weeks because of development of red mange. Signs of ethionine intoxication were not evident before death or at necropsy. Dog 4 received a daily dose of the drug of 10 mg per kilo of body weight for 14 weeks and became anorexic and jaundiced. In addition to the usual fatty liver and atrophic pancreas, necropsy revealed 2 small duodenal ulcers. Dog 5 responded more rapidly, as is expected when the dose of ethionine is 25 mg per kilo of body weight per day, and died of a perforated duodenal ulcer during a histamine test. The dog was jaundiced and had the usual fatty liver.

In general, the changes in the gastric secretory responses from Heidenhain pouches during administration of ethionine were inconstant and variable. Mean values for volume of juice secreted (Table I), concentration and daily output of free hydrochloric acid (Table II) and concentration and daily output of pepsin (Table III) sometimes increased and sometimes decreased during administration of ethionine, and in only a few instances were mean differences statistically significant. No correlation was apparent between any effects of the drug on gastric secretion and either

TABLE III. Mean Concentration and 24-Hr Output of Pepsin in Gastric Juice from Heidenhain Pouches in Dogs before and during Oral Administration of Ethionine.

Dog	Daily dose of ethionine, mg/kg body wt	Pepsin						Mean diff. and stand. error	
		Before ethionine			During ethionine				
		Collections	Units/ml	Units/24 hr	Collections	Units/ml	Units/24 hr	Units/ml	Units/24 hr
1	1	16	215	5255	30	175	6393	−40 ± 23.5	− 1138 ± 1403
	10	16	215	5255	30	197	7684	−18 ± 24.8	+2429 ± 1560
2	2.5	14	115	5197	31	96	4799	−19 ± 11	− 398 ± 657
	10	14	115	5197	35	133	3226	+18 ± 13.1	−1971 ± 534*
3	5	9	254	6950	12	280	9946	+26 ± 54.4	+2996 ± 3315
4	10	12	140	8728	35	105	5948	−35 ± 24.6	−2780 ± 1148*
5	25	15	341	9087	7	259	5487	−82 ± 83.5	−3600 ± 3750

* Mean difference was significant at 5% level.

TABLE IV. Mean Maximal Volume and Concentration of Free Hydrochloric Acid in Gastric Juice Secreted during 15-Min. Periods in Response to Histamine by Heidenhain Pouches in Dogs.

	Before ethionine			After ethionine			Mean difference	
Dog	Collections	ml/15 min.	Hydrochloric acid, mEq./l	Collections	ml/15 min.	Hydrochloric acid, mEq./l	ml/15 min.	Hydrochloric acid, mEq./l
1	5	4.3	154	4	4.2	136	– .1	–18
2	1	12.7	149	5	8.1	147	–4.6	– 2
3	5	9.9	154	2	8.1	156	–1.8	+ 2
4	3	6.1	146	4	4.0	145	–2.1	– 1

the dose of ethionine or the length of time it was given. Likewise, the gastric secretory response to histamine was altered so little during administration of ethionine that the conclusion was that no specific effect was present (Table IV).

Comment. The inhibitory effect of ethionine on pancreatic secretion of amylase, lipase and trypsin appears to be specific and can be demonstrated in acute experiments in dogs before enough time has elapsed for atrophy of pancreatic tissue to occur(2). In contrast to this well-documented effect of ethionine stands the present observation: that secretion of another digestive enzyme, pepsin, as well as secretion of free hydrochloric acid by canine gastric mucosa, is not demonstrably affected by the drug given for relatively long periods and in doses varying from those producing minimal effects to those producing obvious impairment of metabolism.

Conclusions. Ethionine, administered orally to dogs with Heidenhain pouches in daily doses of from 1 to 25 mg/kg body weight for 3 to 23 weeks, did not produce demonstrable change in the secretion of pepsin or free hydrochloric acid obtained in response to feeding or to subcutaneous injection of histamine.

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Some Properties of Yoshida Sarcoma Cells Resistant to Methyl-bis-(β-chlorethyl)-amine N-oxide.* (21519)

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Burchenal *et al.*(1) and Law *et al.*(2) reported development of resistance to folic acid antagonists in transplantable mouse leukemia. The present author(3) has obtained 3 resistant variants of the Yoshida sarcoma to Methyl-bis-(β-chlorethyl)-amine N-oxide (MBAO), by passing tumor cells through animals treated with the agent. Details concerning this agent are to be found in the papers of Ishidate and Yoshida *et al.*(4,5). The study here reported is concerned with 1) effects of other known antitumor agents on the resistant variant and 2) behavior of resistant tumor cells carried in serial transplant in the absence of the agent.

Materials and methods. Rats of mixed breed, weighing about 100 g were used for transmission of the tumor, each receiving intraperitoneal inoculation of 0.02 cc of undiluted tumor ascites, containing about 2 x 10^6 tumor cells; they showed nearly 100% susceptibility to the tumor. The MBAO-s.c. resistant subline of the Yoshida sarcoma, which was resistant to treatment with consecutive subcutaneous injections of daily dose of 10 mg/kg of MBAO, beginning just after inoculation of the tumor, has been used as MBAO-resistant subline. The general characters of this subline have been reported(3). Growth rate of tumor was indicated by the number of tumor cells per cc of ascites, unless otherwise stated, using the method described previously(3).

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Results. A. *Effect of other antitumor agents on tumor cells of the MBAO-s.c. resistant subline.* Methyl-bis-(β-chlorethyl)-amine (Methylbis), Tris-(β-chlorethyl)-amine (Tris), Tris-ethylen-imino-s-triazine (TEM), 8-Azaguanine (guanazolo) and colchicine all show tumor-inhibiting properties. Transfer generations 56 through 58 of the MBAO-s.c. resistant subline were employed to test the effect of these antitumor agents, and corresponding transfer generations of the original susceptible strain of the Yoshida sarcoma served as control material. On the 5th day after inoculation, rats were intraperitoneally injected at the following dosage levels per kg body weight: Methylbis 1 mg, Tris 1 mg, Tem 1 mg, 8-Azaguanine 400 mg and colchicine 40 mg. These doses of the agents, with the exception of guanazolo, were always dissolved in 0.5 cc of 0.85% NaCl solution. 8-Azaguanine was dissolved in 2 cc of 0.01 N NaOH. In each animal injected with colchicine the mitotic figures in 2000 tumor cells were counted just before and 3, 6, 9, 12 and 24 hours after the injection, and metaphase figures were classified into 3 types according to the degree of cell-injury as noted previously(6). It can be seen by reference to Fig. 1 that both increase in number of mitotic figures and degree of cell-injuries were quite similar in two groups. Hence, it may be concluded that the tumor cells in the subline resistant to MBAO showed no decrease of sensitivity to colchicine. 8-Azaguanine did not produce a significant inhibition of growth of tumor cells in either susceptible strain or in the MBAO-resistant subline at dosage level used, and the degree of inhibition of growth in these 2 lines was identical (Fig. 2).

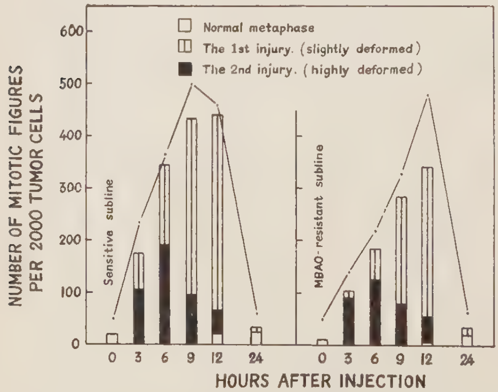

FIG. 1. Effect of colchicine (40 mg/kg) on tumor cells of sensitive and resistant subline of the Yoshida sarcoma. Curves represent total number of mitotic figures/2000 tumor cells; bars represent results of classification of metaphase figures according to degree of injury. Each curve and bar avg of 5 rats.

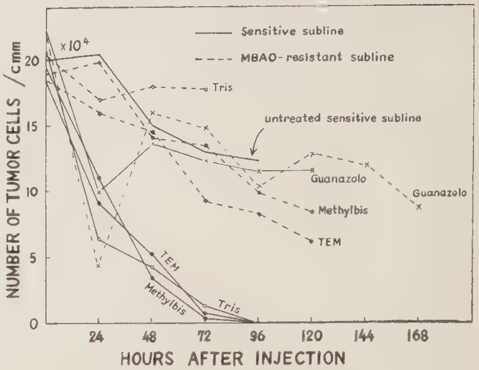

FIG. 2. Effect of Methylbis, Tris, TEM (1 mg/kg) and guanazolo (400 mg/kg) upon tumor cells of sensitive and resistant subline. Each point avg of 5 rats.

In contrast to the observed effects of such unrelated antitumor agents on growth of susceptible and resistant tumor cells, an unequivocal decrease of sensitivity was observed when MBAO-resistant tumor cells were grown in rats treated with closely related antitumor agents, Methylbis, Tris, and TEM. The results are presented in Fig. 2. These 3 agents did not produce a significant decrease in the number of tumor cells in the resistant subline,

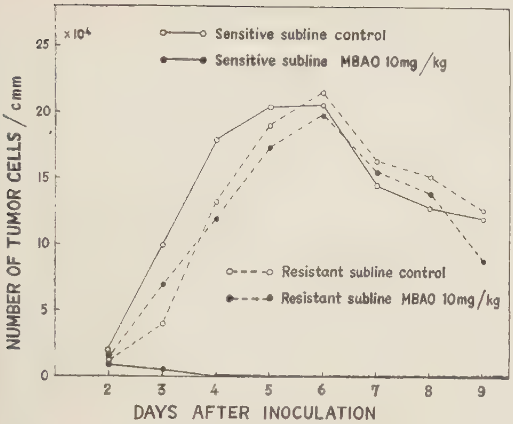

FIG. 3. Behavior of resistant tumor cells carried through MBAO-free host during 24 transfer generations. Each point avg of 5 rats, except for resistant subline treated with MBAO which represents avg of 9 rats.

whereas susceptible tumor cells disappeared almost completely from ascites within 72-96 hours after treatment. At the same time, degeneration and destruction of tumor cells were scarcely seen in the resistant subline, and infiltrative growth of resistant tumor cells was found in the same degree as in the untreated sensitive subline.

B. *Behavior of resistant tumor cells carried in serial transplant in absence of the agent.* The results are presented in Fig. 3. The MBAO-s.c. resistant subline has been carried through 24 successive transfers for about 200 days in host animals not given MBAO. At the 33rd transfer, 24 transfer generations following establishment of this subline in MBAO-free animals, tumor cells were transferred into 9 rats, which were treated with consecutive subcutaneous injections of daily doses of 10 mg/kg of MBAO beginning just after inoculation. No change has been observed in the susceptibility of these cells to MBAO during this period of transfer through MBAO-free hosts.

Discussion. Tumor cells in the MBAO-resistant subline did not show a significant decrease of susceptibility to 8-Azaguanine and colchicine, though the test was done with small doses which produce a relatively slight effect in the susceptible tumor strain. Consequently, it is apparent that tumor cells resistant to MBAO were not cross-resistant to these agents. On the other hand, the resistant tumor cells showed a cross-resistance to other closely related antitumor agents, Methylbis, Tris and TEM. These 3 compounds belong to alkylating agents like MBAO. Burchenal *et al.*(7,8) and Law(9) reported that the resistant variant to one of the folic acid antagonists in mouse leukemia is cross-resistant to all other folic acid antagonists tested.

Summary. A variant of the Yoshida sarcoma which became resistant to Methylbis-(β-chlorethyl)-amine N-oxide (MBAO) upon passage through animals treated with the agent, has been demonstrated to be resistant to 3 other alkylating agents, Methylbis-(β-chlorethyl)-amine, Tris-(β-chlorethyl)- amine and Tris-ethylenimino-s-triazine. It proved sensitive to another unrelated antitumor agent, colchicine. Resistance to MBAO has remained unchanged following 24 consecutive serial transfers in rats free of the agent.

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Autoradiographic Visualization of Ca^{45} Intake by Normal and Pathological Cartilage *in vitro*.* (21520)

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It has been previously demonstrated(3-5), that sections of mineralized tissues placed in a solution of radioactive phosphorus or calcium, could produce thereafter autoradiographs of mineral distribution. This phenomenon was explained by either atomic interexchange(5) or physical adsorption(4).

Following prolonged autoradiographic exposure after treatment with Ca^{45}, other localizations in sections became evident. The radioelement appeared for instance in cartilage, in certain regions of the growing bones and teeth following demineralization, in portions of connective tissue, in the wall of arteries, in mucous glands, in the epidermis and hair follicles, in the retina and other parts of the nervous system. These localizations are more difficult to explain. A survey of different aspects of this phenomenon in relation to cartilage is hereby reported.

Methods. Tissues from various animals, cattle, pig, dog and rat as well as man, were fixed in aqueous formaldehyde or formaldehyde-ethanol, and cut following routine paraffin or celloidin embedding. All calcified tissues were demineralized some in formic-citrate at pH 4.9, some in disodium ethylenediamineteraacetate dihydrate (Sequestrene) at pH 7.6.(6) Sections of 10 μ in thickness were affixed to glass slides and placed for 2 to 4 hours at room temperature, in Coplin jars containing ionized $Ca^{45}Cl_2$ at a concentration of approximately 10 μc per ml. The sections were then rinsed several times in distilled water, dehydrated and covered with a thin coat of celloidin. "Integrated autoradiographs" were made by the improved inversion technic(7,8).

Results. Hyaline cartilage from the trachea in the human (Fig. 1) and from heads of young animals showed a general, diffuse image of the Ca^{45} in the autoradiographs. The concentration of the radioelement was greater in cartilage than in adjacent mucous glands. When the photographic exposure was ideal, a progressive concentration from the periphery towards the center of the cartilage piece could be seen (Fig. 5). Older tracheal cartilage in the human (Fig. 2) showed a progressive decrease in the matrix and an apparent concentration in the lacunae. In mineralized portions of old human trachea, after decalcification, no calcium intake was detectable.

In the heads of long bones, a different picture was visualized in the young rats as compared to the older ones. The young tissue of this type (Fig. 3), showed like in ordinary cartilage, a diffuse distribution of the radiocalcium but with a progressive concentration towards the region of the hypertrophic cells. The ossifying areas showed a decrease by comparison with the general picture. The heads of long bones in adult rats (Fig. 4) exhibited the presence of radiocalcium practically only at the level of the epiphyseal plate. The remnants of cartilage embedded in the bone spicules underneath, showed a minor intake of Ca^{45}. By comparison, the articular surfaces have produced practically no image in the autoradiograph.

A group of specimens of the costochondral junction of infants having suffered from various degrees of rickets, has revealed interesting findings. In all specimens (Fig. 5, 6) there was an intense intake of radiocalcium at the level of the hypertrophic cartilage similar to that observed in normal epiphyseal cartilage. On the other hand, a progressive decrease of the calcium intake was observed in normal young cartilage preceding the above mentioned region of the specimen (Fig. 5).

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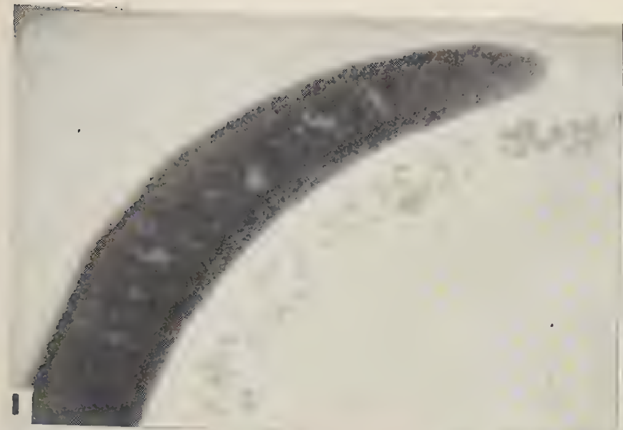

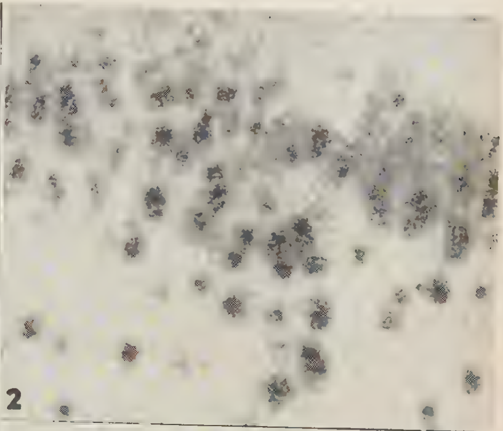

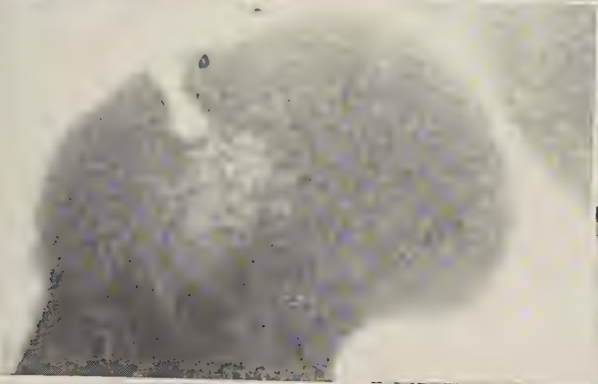

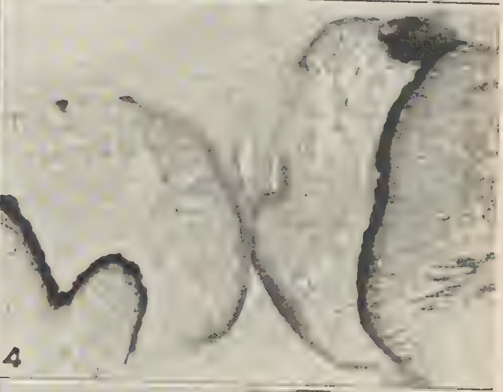

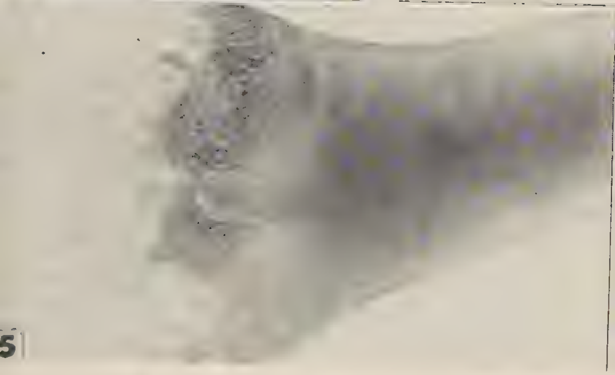

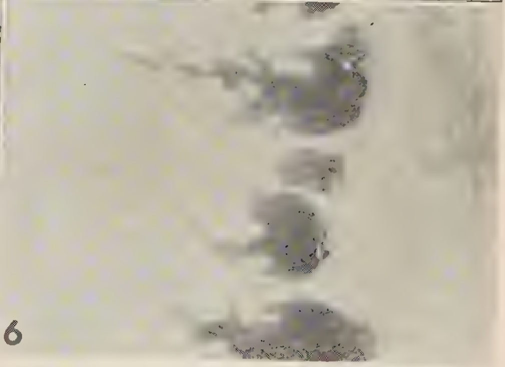

FIG. 1. Portion of trachea of a 6-year-old child. Cartilage and mucous glands show Ca^{45} intake. Unstained integrated autoradiograph, ×27.

FIG. 2. Portion of tracheal cartilage from a woman, age 68. The Ca^{45} intake is visible only over the large lacunae and immediate vicinity of those close to surface. Unstained integrated autoradiograph, ×93.

FIG. 3. Lower extremity of femur and parts of tibia of a 10-day-old rat. U.I.A. ×27.

FIG. 4. Lower extremity of femur and upper extremities of tibia and fibula of adult female rat. Ca^{45} is present almost exclusively in epiphyseal plate and cartilaginous core of bone spicules. Inverted and stained autoradiograph, ×10.

FIG. 5. Costochondral junction of rib of infant suffering from rickets of short duration. Ca^{45} is present all over the cartilage but apparently in larger amounts in hypertrophic area and in central portion of "normal" cartilage. U.I.A. ×10.

FIG. 6. Costochondral junction of rib of child suffering from long standing rickets. Hypertrophic area is strongly positive for Ca^{45} but "normal" cartilage, on right, shows practically no reaction. U.I.A. ×10.

The decrease of calcium intake was progressively more apparent as the rickety lesion was of longer duration (Fig. 6).

To recognize the nature of the chemical substances involved in the above described phenomena, comparable sections were stained by the routine haematoxylin-phloxine-orange procedure, others by the Masson light green trichrome(9), others by toluidine blue for metachromasia(10). Some sections were incubated in 0.1% aqueous hyaluronidase at pH 5.8 and 37°C previous to soaking in the radioactive bath.

Whenever mucus was present it appeared as yellowish-orange with the H.P.O. stain and pale yellow after the Masson trichrome. The normal cartilage was of course, strongly metachromatic after toluidine blue staining. By comparison, the older cartilage which did not retain Ca^{45} stained reddish with H.P.O. and red, mauve or green with the Masson stain. Metachromasia was present but generally decreased over these areas. The hypertrophic zone of the rickety cartilage stained normally in all specimens but the preceding young cartilage was seen to lose its pale yellow colour and appear progressively greener in the Masson stained specimen, as the lesion progressed. A slight decrease in the amount of metachromasia could be detected.

Hyaluronidase incubation reduced the Ca intake and the metachromatic staining of all specimens to a minimum obtained after 3 hours incubation.

Discussion. These experiments seem to indicate that the most important substances involved in Ca^{45} *in vitro* intake by cartilage are the mucopolysaccharides hydrolized by hyaluronidase(11). On the other hand, the distribution of Ca^{45} in Fig. 3 of the present report corresponds perfectly to the autoradiographic distribution of chondroitin sulfate synthesized *in vivo* from $S^{35}04$ as demonstrated by Dziewiatkowski(12) and Bélanger (13). Whether the Ca^{45} has been deposited by atomic interexchange with already present organic bound Ca^{43} or whether we are dealing here with a process of Ca adsorption by the mucopolysaccharides acting as cation binders has not been determined as yet. Theoretical views on the biochemical significance of chondroitin sulfate in relation to calcium based on different experimental approaches have already been expressed by Newman, Boyd and Feldman(14) on the one hand and Sobel and Burger(15) on the other hand. It seems however that the *in vitro* intake of Ca^{45} by cartilage is related to the presence of chondromucin or the mucopolysaccharide fraction thereof and that semiquantitative data on the relative amount of this substance in a given part of cartilage have been obtained by comparative autoradiography. These have presently made evident considerable histochemical differences in the composition of the ground substance of young, old, degenerate and rickety specimens of hyaline cartilage and also between various areas of the head of long bones.

Summary. Demineralized sections of normal and pathological cartilage from various sources, were soaked in a weak solution of $Ca^{45}Cl_2$ and coated with fluid photographic emulsion. The autoradiographic record has shown an apparently specific pattern of Ca^{45} intake. Comparisons of these images and those obtained with $S^{35}04$ *in vivo* and also the negativating effect of hyaluronidase seems to indicate a relationship between Ca^{45} intake and the relative local distribution of sulfated mucopolysaccharides in the tissue.

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Immunity To Visceral Lymphomatosis in Chicks Following Injection of Virus into Dams. (21521)

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Avian visceral lymphomatosis, a contagious, neoplastic disease, is one of the most destructive of all diseases affecting chickens. It may spread by direct or indirect contact and through the fertile egg. This disease may be reproduced readily by filtrates of lymphomatous tumors, plasma, oral washings or feces from cases occurring naturally or induced by inoculation(1-4). Furthermore, chicks may be infected by several routes(5), including aerogenic, conjunctival, nasal, oral, cloacal, and by parenteral inoculation. Survivors of transplants of the lymphoid tumors are immune to reimplantations(6-8), and sera from such birds were found to have cytotoxic effect(9) on the tumor cells. However, the same chickens may later develop the disease, *i.e.*, lymphomatous involvement of the viscera, by action of the virus(10). Treated and untreated tissue vaccines have failed to give protection against the disease(11).

Presumptive evidence that the viral agent causing visceral lymphomatosis may provoke the formation of protective antibodies and that these antibodies may be transmitted to the progeny in significant proportions, was recently obtained in studies on the incidence of the disease in the progeny of carrier hens(12) and in attempts to produce the disease in chicks of hens repeatedly injected with the active virus(13).

This paper reports an experiment in which the resistance of chicks to challenge inoculation with the virus of visceral lymphomatosis was greatly increased by giving the dams repeated injections of live virus preparations.

Materials and methods. A group of 14 hens received a series of injections of an inoculum containing the virus of visceral lymphomatosis. The relative resistance of the progeny of these hens, hatched of eggs laid before and after the immunizing period, was determined by inoculation with 3 serial dilutions of a virus preparation of known potency. The hens were of an inbred line of S. C. White Leghorns which had been selected for susceptibility to lymphomatosis and which had been maintained in isolation for the past thirteen years to reduce the extent of natural infection. They remained in the same quarters while they laid the eggs used in three biweekly hatches, the chicks of which were used in three challenge inoculations of the "before" period. Immediately following this period, the hens were moved to a pen by themselves but in a location somewhat less isolated from other chickens than previously, and the immunizing injections were begun. Two preparations were used as immunizing materials. Both were live virus preparations of lymphomatosis which had been passed through Selas O_2 porcelain filter candles and stored in a CO_2 icebox until used for the injections. Preparation L13 was from lymphomatous livers of the fourteenth serial passage of Strain RPL 12 (14), and the second preparation, L16, was from similar material of the sixteenth serial passage. The hens were 2½ years of age when the injections were begun. They were injected intravenously at the rate of 0.1 ml on alternate days the first week, 1.0 ml 3 times weekly for the next three weeks, and then one injection of 1 ml 10 days after the last injection of the weekly series. The virus preparation, L13, was used for injections made during the first 2 weeks and L16 was used for all other injections. The collection of hatching eggs for testing the relative resistance of chicks hatched of eggs laid during the "after" period, began 7 days after the last immunizing injection and continued for a period of 6 weeks, providing sufficient eggs for three hatches and chicks for resistance test inoculations. All chicks were pedigree hatched and the chicks from each hen of each hatch were distributed as equally as possible among each of three dosage lots. Thus, the progeny from all hens were distributed equally between the

TABLE I. Response to Challenge Inoculation with 3 Doses of Virus of Visceral Lymphomatosis of Chickens Hatched from Eggs Laid before and after Hyperimmunization of the Dams.

	Dose of virus inoculated in log g tumor								
	−3			−5			−7		
Chicks of eggs laid	No. chicks inoculated	% visc. lympho.	Avg age at death	No. chicks inoculated	% visc. lympho.	Avg age at death	No. chicks inoculated	% visc. lympho.	Avg age at death
Before hyperimmunization	64	70.3	64.4	60	56.7	111.6	63	41.3	137.6
After hyperimmunization	39	12.8	109.4	37	5.4	192.0	37	2.7	190.0

3 dosage lots. The chicks were inoculated intraperitoneally with 0.2 ml of 3 serial 100-fold dilutions of the virus preparation previously described and designated as L16. These three dilutions of the tumor filtrate represented a dose of log -3, -5, and -7 g of tumor. Chicks inoculated with different dosages were maintained in separate isolation cubicles for the first 75 days of life(3) after which they were transferred to large pens where several groups were maintained together. The birds were maintained for an experimental period of 200 days. All birds that died during this period were examined and a diagnosis was made on the basis of gross alterations.

Results. A summary of the results of the foregoing experiment is presented in Table I. These data show that chicks hatched of eggs laid by the 14 hens were quite susceptible to inoculation. Of 64 chicks that had received an inoculum equivalent to 0.001 g of lymphomatous liver, 45 died in an average of 64.4 days with lymphomas and the two 100-fold dilutions of this dose gave lower tumor rates in proportion to the dose injected. In contrast, only a few of the chicks which hatched of eggs laid after the dams had been hyperimmunized, died of tumors after inoculation.

The quantitative relationships between dose and response were similar in the "before" and "after" groups and are similar to other titrations (unpublished) in that changes in dose resulted in only small changes in response, thus the relationship was represented by a very flat curve. Because of this relation, one cannot expect complete absence of tumors even at very low dosages.

It is recognized that non-immunized hens were not included in this experiment; however, it is not probable that the resistance of the chicks could have changed without exposure of the hens to the virus in as much as the hens were 2½ years of age at the time of the "before" test and there was an interval of only 3½ months between the "before" and "after" tests. A significant change in the potency of the virus preparations during this same period is also not probable since extensive titrations of the same preparations showed that during an 18 month storage period there had occurred a reduction in the LD50 of only about ½ log (unpublished data).

These marked differences within each dosage group between the "before" and "after" chicks, suggests an increase in resistance to the disease, resulting from repeated injections of the dams with live virus, of a magnitude and duration sufficient to prevent or abort the establishment of active infection with the challenge virus. It would seem logical to suggest that these results were due to a stimulation of antibody formation in the laying hens and depositions of this antibody in eggs which resulted in a passive immunity in the chicks. The production of a specific passive immunity in chicks as a result of vaccination of hens, or a recovery from the disease, has been adequately demonstrated in several other viral diseases of poultry(15,16).

Although many investigators have reported evidence for antibodies to the causative agents of other avian neoplasms (fibrosarcomas and erythromyeloblastosis), the experiment reported herein provides the first direct evidence that a significant immunity may be provided by the virus of visceral lymphomatosis and indirect evidence that it is due to serum antibodies of the usual type.

Summary. Results obtained in experiments involving 14 immunized hens and 300 test chicks indicate that the virus of visceral lymphomatosis is capable of inducing antibodies in adult chickens, and that these antibodies are transmitted to the chick via the egg, giving the former a significant immunity to challenge inoculation.

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Effect of X-irradiation on Purine-Metabolizing Enzymes of Rat Liver and Spleen.* (21522)

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Evidence obtained by various technics has indicated that nuclear structures of cells, rather than cytoplasmic, are principally affected following exposure of animals to whole-body irradiation. Elucidation of the biochemical changes that occur in the nucleus after irradiation may eventually lead to a better understanding of the biological effects of radiation. Studies of this type, however, are limited by the meagre information available on the fundamental biochemistry of the nucleus. Hogeboom *et al.*(1) have summarized the biochemical properties which can be attributed to the rat liver nucleus; they are astonishingly few, but include the DPN-synthesizing mechanism of the cell. Potter and Grannis(2) reported that this synthetic system is also localized in nuclear fractions isolated from rat spleen and was unaffected by whole-body X-irradiation. Recently, with the use of a modified Behrens procedure, it was shown that the purine-metabolizing enzymes, adenosine deaminase (AD) and nucleoside phosphorylase (NP), are concentrated in the nucleus of the calf liver cell(3); neither of these enzymes was found in either calf or rat liver nuclei when the latter were separated in sucrose solution, in confirmation of an earlier report(4). The conclusion was that these soluble enzymes are present originally in rat liver nuclei and are washed out during preparation in aqueous media. It was thus of interest to determine whether whole-body X-irradiation had any effect on the activities of these 2 liver enzymes, particularly the phosphorylase, since the latter may play a role in the synthesis of purine ribosides prior to nucleic acid formation(5). Spleen NP activity was included in this study although no evidence has been presented bearing on the intracellular localization of the enzyme in this organ.

Methods. All of the experiments utilized male rats obtained from the Wistar Institute. The animals employed for the AD tests weighed between 190-250 g, while those used in all other studies weighed between 90-130 g.

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TABLE I. Effect of X-Irradiation on Adenosine Deaminase and Nucleoside Phosphorylase Activities of Rat Liver and Spleen.

Enzyme	Organ	Avg specific activities* with ranges at varying days after irradiation					
		1	2	4	7	8	10
Adenosine deaminase	Liver						
Control		.0119† (.0110-.0127)	.0118 (.0114-.0130)			.0119 (.0107-.0128)	.0102 (.0092-.0108)
X-Irrad.		.0110 (.0100-.0126)	.0105 (.0089-.0117)			.0104 (.0102-.0106)	.0102 (.0088-.0108)
Nucleoside phosphorylase	Liver						
Control		.416 (.392-.437)	.355 (.318-.400)	.385 (.353-.414)	.391 (.342-.479)		.341 (.314-.363)
X-Irrad.		.380 (.355-.400)	.382 (.372-.395)	.386 (.363-.426)	.403 (.323-.483)		.320 (.300-.332)
Nucleoside phosphorylase	Spleen						
Control		.460 (.429-.488)	.473 (.415-.525)	.518 (.439-.559)	.527 (.503-.566)		.513 (.473-.560)
X-Irrad.		.618 (.401-.740)	.739 (.597-.851)	.772 (.642-.918)	.826 (.680-.962)		.716 (.635-.849)

* For adenosine deaminase = micromoles adenosine deaminated/min./mg N. For nucleoside phosphorylase = micromoles uric acid formed/min./mg N.

† Each value represents avg of 4 animals.

The rats were given, in a single exposure, a total dose of 600 r of 240 KV X-rays filtered through 1.0 mm Cu and 1.0 mm Al at an average rate of 66.8 r per minute and a target distance of 50 cm. The animals were generally irradiated in groups of 4 to 6 in metabolism cages. Usually, food was withheld from exposed and control rats for the first 24 hours after irradiation. A stock commercial diet (Nurishmix) was then made available to both groups. The control animals were always carefully pair-fed against the exposed to correct for any changes in enzyme activity that might be caused by the decreased food intake of the irradiated rats. Water was provided *ad libitum* throughout the experiments. Control and X-rayed animals were sacrificed by ether anesthesia, and the livers and spleens were removed, washed free of adhering blood, and homogenized in a Ten Broeck glass homogenizer. Since the activities of both enzymes were found to be little changed whether the homogenates were prepared in 0.25 M sucrose or water, the latter was used as a suspending medium. The livers were first homogenized 1:10, and the spleens 1:25, in cold glass-distilled water. For the AD tests, the liver homogenates were further diluted to 1:50; for the measurement of NP, 1:250 dilutions of the liver and spleen homogenates were made. All assays were carried out immediately at 2 levels of homogenate (0.1 and 0.2 ml for AD and 0.05 and 0.1 ml for NP) at a temperature of 24.6°C. The activities were directly proportional to the concentration of tissue used. Both enzymes were studied essentially according to the spectrophotometric principles outlined by Kalckar(6), but the components of the test systems were used at levels recommended for mouse liver homogenates(4). AD activity was followed by the disappearance of the 265-mμ band of adenosine. In the NP asay, inosine is split to ribose-1-phosphate plus hypoxanthine, and the latter, in the presence of xanthine oxidase (Worthington Biochemical Corp.), is oxidized quantitatively to uric acid, which was measured by its appearance at 292.5 mμ. In preliminary experiments it was found that under the conditions used here spleen NP activity was the same with or without cyanide (used in the liver assay to inhibit uricase). Therefore, cyanide was omitted from the test for spleen. The specific activities were calculated by the use of the optical constants reported by Kalckar(6,7). For the deaminase assays,

TABLE II. Effect of X-Irradiation on Wet Weight, Nitrogen Content, and Total Nucleoside Phosphorylase Activity of Rat Spleen.

Wet wt*		Nitrogen content*		Total nucleoside phosphorylase activity†	
C‡	X	C	X	C	X
347 (254-534)	152 (75-278)	9.43 (6.89-14.59)	4.24 (2.05-8.04)	4.671 (2.882-6.484)	3.040 (1.550-6.123)

* Values represent avg spleen wet wt and avg spleen N content in mg. Ranges in parentheses.

† Values represent avg spleen total enzyme activity (micromoles uric acid formed/min./spleen).

‡ C = control; X = irradiated.

readings were taken at 5 minute intervals for 20 to 25 minutes; 1 minute readings were taken for 5 to 7 minutes for the phosphorylase tests. Both reaction rates were essentially linear during these periods. Total nitrogen was determined by the micro-Kjeldahl method.

Results. From the data presented in Table I, it can be seen that no significant change occurred in liver AD activity in animals sacrificed on the first, 2nd, 8th, and 10th days following exposure. The average specific activities of the 2 groups of rats over the 10-day period were: irradiated, 0.0105; control, 0.0116. The activity of liver NP was also unaffected in animals studied on the first, 2nd, 4th, 7th, and 10th days after irradiation (Table I). However, the specific activity of spleen NP (Table I) of irradiated rats was found to be considerably increased over that of the control animals. The increase ranged from 35 to 57% during the 10-day period with an average value of 48%. The average spleen specific activities for the two groups of rats during the 10 days were: irradiated, 0.734; control, 0.498. When this experiment was repeated 4 months later under similar conditions, there appeared to be a lag in the rise of enzyme activity over the control level. The increases in activity on the first, 2nd, 5th, and 7th days following irradiation were 13, 16, 35, and 50%, respectively. Six control and 6 X-rayed animals were sacrificed on the first 3 days and 2 from each group on the last day. The average spleen specific activity for 20 control animals was 0.494, in excellent agreement with the value of 0.498 found for the 20 control rats listed in Table I. It is of interest to note that under the experimental conditions used here, NP activity of normal spleen was consistently greater than that of normal liver by 30%.

Several experiments were performed to determine whether irradiation resulted in the formation (or release) of a NP activator or the destruction of a NP inhibitor. The activity of combined control and irradiated homogenate was additive and thus offered no evidence for either of these possibilities.

In addition to expressing results on the basis of the activity per mg of nitrogen (specific activity), data were tabulated for the activity per whole spleen (total activity). In accord with the observations of other investigators(8-10) it was found that the spleens of irradiated animals lost considerable weight (Table II). For the first NP experiment described above, the average spleen weight loss was 55% on the basis of both wet weight and nitrogen content. Because of this marked decrease in spleen nitrogen the average spleen total NP activity was actually reduced by 35% over the entire post-irradiation period (Table II). This reduction was apparent on the first day after exposure.

A time study was made on another series of rats in an effort to determine how soon following irradiation the increased specific activity could be detected. Prior to exposure, 6 control animals were sacrificed and spleen NP levels were determined to give a zero time, or base, activity. Spleen homogenates prepared from X-rayed animals (4 at each interval) were assayed at 2, 5, 8, 11, 24, and 48 hours after exposure. The first detectable rise in activity occurred at 11 hours and amounted to 20%. At 24 and 48 hours, the enzyme activity increased to 25%. While the change in activity was smaller in this series of irradiated rats, the onset of the rise was in good agreement with the data obtained in the first NP experiment. Here, the studies performed on the first day following irradiation were actually made on animals sacrificed 13-18 hours after exposure.

Discussion. It is clear from the data obtained with rat liver homogenates that neither of the purine-metabolizing enzymes reported to be localized in the rat liver nucleus is affected by 600 r of total-body X-irradiation. The increased activity of one of these enzymes of spleen, nucleoside phosphorylase, is of interest in view of the reports that 5-nucleotidase(10,11), ATPase(10,11), and β-glycerophosphatase(10), are also increased in activity in spleen homogenates, but to a much greater degree, following irradiation of the intact animal. However, not all spleen enzymes are similarly affected. As in the case of liver, several respiratory enzymes(10) are unchanged by whole-body irradiation, while the process of oxidative phosphorylation is markedly diminished(10,12).

Summary. Whole-body X-irradiation (600 r) had no effect on the activities of rat liver adenosine deaminase and nucleoside phosphorylase studied at intervals over a 10-day period following exposure. The specific activity of spleen nucleoside phosphorylase increased, although the total organ enzyme activity was reduced as a result of the decrease in spleen size.

The assistance of Mrs. Ellen A. Thompson is gratefully acknowledged.

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Biological Properties of a Highly Purified Preparation of 17-Alpha Estradiol.

(21523)

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The biological activities of β-estradiol have been described fully, whereas those of the 17 α-isomer are not. This paper deals with an examination of certain biological properties of 17 α-estradiol. With regard to its estrogenic action, Schwenk and Hildebrandt(1) reported α-estradiol to have one-fourth the activity of β-estradiol. Later, Whitman, Wintersteiner and Schwenk(2) using a more highly purified preparation indicated α-estradiol to have approximately one-twelfth the activity of the beta form. In the same publication, reference was made to unpublished experiments of Hirschmann and Wintersteiner which indicated that the activity of the alpha isomer was less than one-twelfth that of β-estradiol. Thayer *et al.*(3) reported bioassay results on a purified preparation of α-estradiol and its benzoate for estrogenic activity, and showed that relative to β-estradiol the activity of α-estradiol varied approximately from one-seventh to one-fiftieth the activity, depending on the method of bio-assay. Recently Goldzieher *et al.*(4) reported the "estrogenically in-

TABLE I. Results of Bio-assay of Alpha-Estradiol in Female Mice. Six observations in each series.

	α-Estradiol				Standard; β-Estradiol = 100%			
Dose (μg/mouse/day)	8	11	15	20	0.008	0.011	0.015	0.020
Mean body wt (g)	7.6	6.4	7.3	7.8	6.6	7.6	7.8	6.9
" uterus wt (mg)	18.3	17.5	20.6	23.1	10.3	16.6	15.8	17.2

ert estrogen" (α-estradiol) to be effective in causing proliferation of atrophic skin. These investigators suggested that the effect might be associated with an anabolic action.

Because it was possible that α-estradiol might exert significant anabolic action and yet be extremely weak or devoid of estrogenic action, a very highly purified preparation of this steroid was obtained,* assayed for estrogenic activity, and then evaluated as a protein anabolic and pituitary gonadotrophin inhibiting agent. It was also possible, during these studies, to obtain certain additional data on the estrogenic and androgenic activities of the compound.

Methods Anabolic and androgenic responses were studied using the levator ani, seminal vesicle and ventral prostate responses in immature rats(5); estrogenic activity by the effect on the immature mouse uterus and, secondarily, by the ratio of the seminal vesicle/prostate weights, and pituitary gonadotrophin inhibition in parabiotic rats(6).

Results. A. Estrogenic activity. Based on preliminary assays, the α-estradiol sample was assigned a provisional potency of 0.1% of authentic β-estradiol (=100%) which was used as a reference standard. This information was then utilized in the design of the 8-point assay detailed in Table I. The assay was performed as follows: Using a table of random digits(7), 48 immature female mice of the Carworth CF_1 strain, weighing 7 ± 1 g, were allocated to 24 cages in such a way that each cage finally contained 2 randomly paired mice. The 8 experimental treatments (4 dilutions in corn oil of each isomer) were assigned at random to the 24 cages. Three cages (6 mice) therefore received each treatment. The animals were injected subcutaneously once a day for 3 days. All doses are expressed as micrograms of compound per mouse per day. On the day following the last injection, the animals were weighed, sacrificed and the uteri excised. After removal of adherent tissue, each uterus was weighed to the nearest 0.5 mg on a Roller-Smith torsion balance. Table I presents the mean body weights and mean uterine weights calculated from the experimental results.

The relative potency of the α-estradiol sample as a percentage of the standard (R%) was calculated to be 0.24%. The 95% fiducial limits of this estimate, obtained by Fieller's theorem(8), are $R_L\% = 0.16\%$ and $R_U\% = 0.80\%$. Thus, it may be asserted with 95% fiducial probability that this sample contains not less than 0.16% of β-estradiol activity nor not more than 0.8% β-estradiol activity, with the most likely value being 0.24%. The calculated index of assay precision λ, was 0.277.

B. *Anabolic and androgenic activities.* Having established the possibility of contamination of the α-estradiol with approximately 0.3% β-estradiol, a test for anabolic activity was carried out. Table II presents the data obtained in 2 experiments. It will be noted that neither α-estradiol in daily doses of 1.0-2.5 mg nor β-estradiol at 1.0 mg exhibited any marked effect on the levator ani complex. On the other hand, a daily dose of 0.03 mg testosterone proprionate caused a marked increase in the weight of the muscle. It is also to be noted that the estrogens caused an increase in seminal vesicle weight but not that of ventral prostate, an indication of estrogenic action. It has been known for some time that estrogens increase the weight of the seminal ves-

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* We are indebted to Mr. P. Kabasakalian of the Physical Chemistry Department for purifying the material. Physical constants for the material are: m.p. 223.9-224.9°C. (corr.), rotation (dioxane) $[\alpha]_{20°C} = +50.5°$, $E^{m}_{232} = 2{,}030 \pm 30$ (methanol). Impurity, based on solubility analysis up to 2%; based on countercurrent distribution up to 6%.

TABLE II. Effect of Alpha-Estradiol on Levator Ani and Accessory Organs in Immature, Male, Castrate Rat.*

Exp.	Compound	mg/rat/day	No. rats	Final† body wt (g)	Levator ani† (mg)	Ventral prostate† (mg)	Seminal vesicle† (mg)
I	Corn oil	.2 ml	7	100.5	22.3	7.8	11.9
	α-Estradiol	1.0	7	101.2	35.1	10.5	34.9
	Idem	2.5	7	99.0	29.3	8.9	36.8
	β-Estradiol	1.0	8	87.9	30.3	11.2	34.8
	Test. prop.	.03	7	102.3	82.8	20.5	58.6
	Idem	.05	7	103.2	93.4	29.9	64.1
	"	.075	7	101.9	75.2	45.4	85.5
II	Corn oil	.2	4	95.6	18.0	—	10.1
	α-Estradiol	1.0	4	88.8	29.9	—	25.4
	β- "	1.0	4	81.8	18.0	—	26.2
	Test. prop.	.05	4	92.5	51.5	—	73.8
	Idem	1.0	4	93.0	71.3	—	111.5

* All compounds injected s.c. in 0.2 ml corn oil. Day of castration was first day of injection. Animals were inj. for 7 successive days and autopsied on eighth day.

† These weights are avg weights for group.

icles but not the ventral lobe of the prostate.

C. *Pituitary gonadotrophin inhibition.* The activity of α-estradiol as a pituitary gonadotrophin inhibitor compared with β-estradiol was studied in parabiotic rats, and the results are given in Table III. It will be noted that the minimal effective dose, as measured by the suppression of ovarian hypertrophy, of the α-estradiol appears to be $>1<5$ mg per day compared to a very crude estimate for the β-estradiol of $>0.005<0.008$ mg per day. Roughly, α-estradiol appears to have about one-two hundred fiftieth the activity of the β-epimer.

Summary. 1. The anabolic, the estrogenic and the pituitary gonadotrophin inhibiting actions of a highly purified sample of α-estradiol have been studied in experimental animals. 2. It appears that α-estradiol has no significant anabolic action and that the weak estrogenic and gonadotrophin inhibiting activities might be due to contamination with a very small amount of the potent β-epimer. 3. If the activity found in this preparation is truly due to α-estradiol, then it would appear that α-estradiol has one-two hundred fiftieth the gonadotrophin inhibiting activity of β-estradiol and approximately one-three hundred fiftieth the estrogenic activity.

TABLE III. Effect of Alpha-Estradiol as a Pituitary Gonadotrophin Inhibitor.

Exp.	Compound	Dose, mg/day	No. pairs	Avg organ wt, mg	
				Ovary*	Uterus†
I	Corn oil	—	3	139	51
	Estrone	.5	3	42	91
	α-Estradiol	10.0	5	28	81
II	Corn oil	—	4	135	47
	β-Estradiol	.008	5	82	146
	α-Estradiol	5.00	5	67	83
III	Corn oil	—	2	91	70
	β-Estradiol	.01	2	53	130
		.005	2	77	43
	α-Estradiol	1.00	2	133	56

* Ovaries of left, intact partner.

† Uterus of right, ovariectomized, injected partner.

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A Mechanical Heart-Lung Apparatus with Gas Dispersion - Centrifugal Aerator.* (21524)

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In order to oxygenate blood rapidly enough *in vitro* to meet mammalian needs at 37°C a large interface between blood and aerating gas is mandatory. This condition is presently being met in non-biological oxygenators in two general ways. Either(1) the blood is caused to form a thin film on some large surface(1-9), or (2) the aerating gas is dispersed as bubbles throughout the mass of blood(10-16). Because of its great efficiency as a means of creating a large blood-gas interface, the method of gas dispersion using very minute bubbles appears attractive. An apparatus for this purpose is herein described.

Apparatus. The pump-oxygenator consists of 4 parts: (1) a withdrawal pump to remove blood from the animal's vascular system and to deliver it to (2) an oxygenator, (3) a defoamer to separate and remove excess and waste gases from the blood, and (4) an arterial pump which returns the processed blood to the animal. Each of these parts will be described separately.

Pumps. A modification of the roller pump described by DeBakey(17) and employed extensively by Gibbon and co-workers(4,7) is used at both ends of the present equipment (Fig. 1). The arc of compressible tubing against which the 2 rollers play in their circular excursion is approximately 210°. Thus at least one roller is always compressing the tubing, and prevents any reflux of fluid. There are no valves in the entire apparatus. The same valve effect can be obtained by using three or more rollers with a small arc, but increasing the number of rollers tends to hamper the filling of the tubing. Pump speed is manually controlled by a torque-converter electric motor combination.

Oxygenator. Aeration of the blood is effected by injecting oxygen as minute bubbles into the blood in the oxygenator, a method introduced by Clark, Gollan and Gupta(10). The oxygenator (Fig. 2) consists of (1) a hollow cylindrical microporous porcelain bacteriological filter (Selas Corporation of America). The filter is 6 inches long and the mean pore size is 5μ. It resides within (2) a cylindrical glass jacket. The glass jacket has a side arm for admission of oxygen from a tank. The lumen of the filter contains a current of unoxygenated blood delivered from the animal by the withdrawal pump. A spiral ribbon of polyethylene within the lumen of the filter (Fig. 2) causes the blood to flow in a swirling course. Thus a thoroughly homogeneous mixture of unoxygenated blood and gas bubbles results. Oxygenation is very rapid, and simultaneously carbon dioxide diffuses into the excess of gas bubbles which are introduced intentionally. It is possible to aerate over four liters of blood per minute with this oxygenator in consequence of the tremendous blood-oxygen interface. It can be calculated that were one ml of oxygen gas broken down into bubbles whose size was of the order of magnitude of 5μ, as is done by the filter, the total surface area would approximate 1.2 m^2. In the operation of the filter the inflow of oxygen is so regulated that each liter of blood is mixed with 100 ml of oxygen (approximately 50 ml for saturation of the hemoglobin and roughly 50 ml excess into which carbon dioxide can diffuse). This quantity of oxygen would yield 120 square meters of blood-gas interface. A liter of blood spread out over this surface area would have a thickness of only 8.3 μ. It should be pointed out, however, that a liter of blood is not actually continuously exposed to a surface area of this extent in the extracorporeal circuit. There is some lessening of surface area with time since (1) some of the oxygen disappears as it combines with hemoglobin and (2) bub-

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† Public Health Service Research Fellow of the National Heart Institute.

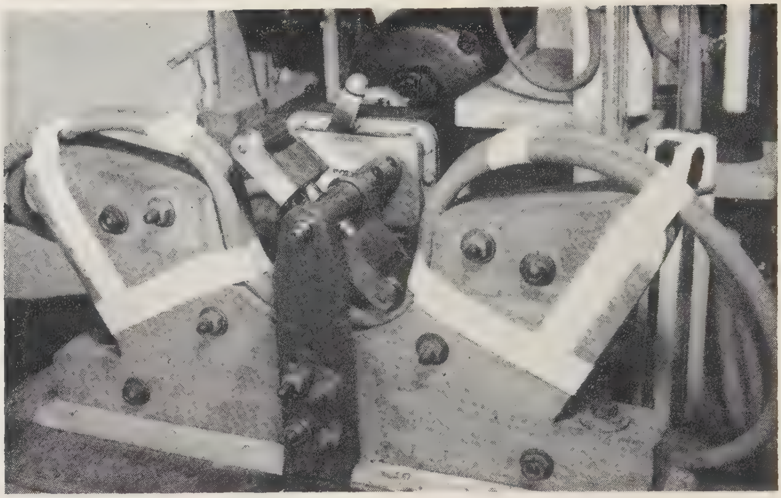

FIG. 1. Arterial pump.

bles coalesce into larger aggregates which tends to diminish the total surface; nevertheless the latter certainly is very extensive. The oxygen coming to the oxygenator jacket is passed through 0.9% saline to be moistened. Saline is used rather than water since the vapor pressure of the water in saline more nearly approximates the vapor pressure of water in blood. Blood is prevented from entering and blocking the filter pores by a device which never permits the jacket pressure to fall below the pressure of the blood within the lumen of the filter. The two pressures are transmitted to opposite sides of a rubber membrane which partitions a small metal-walled chamber into 2 separate spaces. When blood pressure exceeds jacket pressure the membrane deviates, and closes an electric circuit. This actuates a solenoid valve in the oxygen line and admits gas from the tank to the jacket raising the pressure there. The membrane is forced back, and the solenoid shuts and remains so until the jacket pressure again falls below the blood pressure. The device is useful when it is desired to circulate blood through the apparatus without oxygenating it, as in priming the machine. When blood is being oxygenated, the solenoid is constantly held open by closing another switch. The flow rate of oxygen is controlled by varying the pressure in the oxygenator jacket. This provides a convenient way of controlling the amount of carbon dioxide which is lost by the blood in its transit through the oxygenator. The total amount of gas inflow controls the amount of excess oxygen as bubbles carried in the blood stream, and since more carbon dioxide diffuses out of the blood when the excess of bubbles is great, the carbon dioxide loss can be controlled simply by varying the jacket pressure. The blood after receiving its oxygen via the filter passes to the defoamer where the excess gas is separated out.

Defoamer. A great danger in oxygenation by gas dispersion is the creation of foam which must be removed before returning the

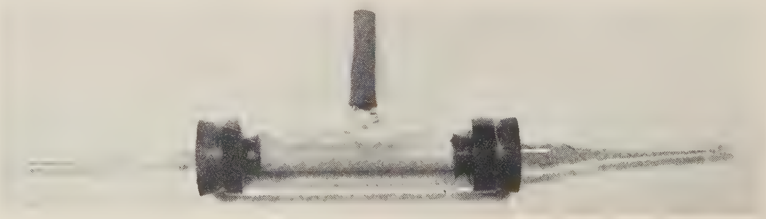

FIG. 2. Oxygenator.

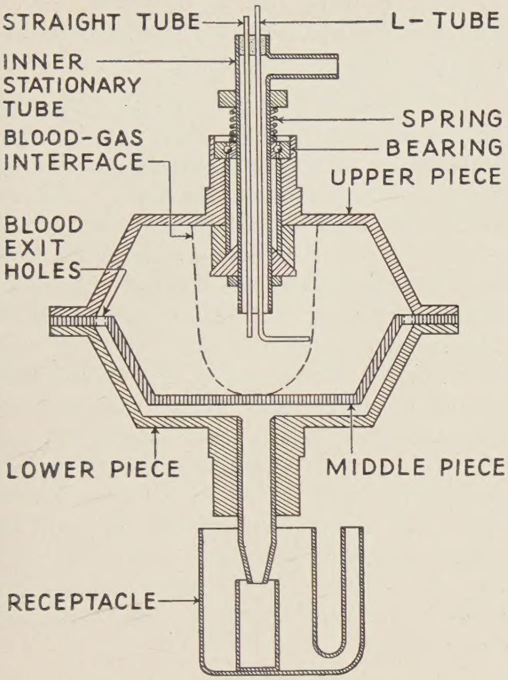

FIG. 3. Cross section of centrifugal defoamer.

blood to the animal. Adequate removal of the excess gas implies (1) that the gaseous phase be separated from the blood phase, and (2) that the foam, after its separation be destroyed at a rate equal to or greater than the rate of its formation. If this second condition is not met, the circulating blood volume and especially the surface active proteins and lipids will gradually be displaced into foam and lost. For defoaming, the present centrifugal method has proved more practical and safer than other methods investigated which included: simple gravity bubble traps, serial filtration through fine filters, air jets to break the foam, use of helium-oxygen mixtures to make the aerating gas lighter in weight, and the use of surface active chemicals such as higher alcohols, ethers, and silicone antifoaming compound. In our hands the present defoamer is the only one found to have sufficient capacity to defoam the rather sizable bubble excess introduced to eliminate optimal amounts of carbon dioxide from the blood.

The constant-flow centrifuge used to separate out the excess gas is shown in cross-section in Fig. 3. It is constructed of aluminum and has a volume content of 1500 ml.

Blood arrives from the oxygenator by way of the inner stationary tube in the top piece. At the bottom of this tube there is an air-tight but movable seal between the center tube and an outer tube which rotates with the body of the centrifuge. This air-tight seal is essential since slight positive pressure is necessary to drive the blood through the centrifuge. In the centrifuge, the blood seeks the periphery and the gas bubbles are forced to aggregate at the center. Since each bubble is bounded by a film which is essentially a thin layer of liquid, and since all of the liquid whether it be in the body of the blood or around a bubble as film seeks the periphery of the centrifuge, it is clear that centrifugation tends to destroy by collapse, as well as to separate bubbles. It is this effect of destroying bubbles that makes this system efficient. After blood and gas have been separated, the blood is forced through the peripheral holes in the middle piece (Fig. 3) into the interval between the middle piece and the lower piece. From this space it is driven down the lower center tube into the glass receptacle below. From the receptacle the blood is returned, bubble free, to the animal via the second return pump.

Waste gases leave the centrifuge via the small centrally placed glass L-tube which also serves to fix the position of the blood-gas boundary, and so keeps the fluid volume in the centrifuge constant. A small current of oxygen enters the centrifuge via the central straight glass tube and with waste gases from the blood leaves via the L-tube. Entering blood tends to shift the blood-gas boundary centripetally. As soon as the latter encroaches upon the end of the L-tube and blocks the escape of gas, pressure is built up. This pumps blood out of the centrifuge and causes the blood-gas boundary to recede away from the end of the L-tube until the gases can again escape. Incoming blood again moves the blood-gas boundary inward, and the cycle repeats itself. In this fashion the volume within the centrifuge can be kept constant to within 75 cc. During the pressure phase when the end of the L-tube is blocked by fluid, a very small amount of foamy blood is forced out of the L-Tube. This is returned to the machine via the glass receptacle (Fig. 3) after passing through a simple bubble trap. The remaining extracorporeal blood volume is held constant by manually regulating the speed of the pumps so that a fixed fluid level is maintained in the glass receptacle.

The receptacle into which the centrifuged blood flows consists of two concentric cylinders (Fig. 3) closed at the bottom and open on the top. The blood flows first into the smaller central glass cylinder where its rotational motion is lost. Then it flows over the side of the latter into the outer cylinder whence it is conveyed to the animal by the return pump. Tubing used throughout the apparatus is Tygon except the pump tubing which is rubber. Cannulae from animal to machine are polyethylene.

It might well be asked whether or not this mode of foam separation is unduly traumatic to cells. In this regard it should be pointed out that the blood is both introduced and withdrawn at the center of rotation where the linear velocity of any particle is small despite a high angular velocity. It is also true that when a particle moves outward to the periphery of the centrifuge where its linear velocity becomes high, it moves with the surrounding blood and with the centrifuge as a unit, *i.e* there is always very little motion of a particle of blood relative to its immediate surroundings. With the apparatus in actual use with dogs hemolysis yielded plasma hemoglobin levels in the range of 150 mg % after 30 minutes of perfusion. After 1½ hours of perfusion plasma hemoglobin sometimes reaches values of 600-700 mg %. Precise data will be published in relation to detailed studies of the animal. Further steps designed to reduce hemolysis will be taken. These include (1) coating the aluminum body of the centrifuge with an inert plastic, (2) introducing radial vanes to diminish still further the relative motion of blood layers, and (3) the determination of a minimal yet adequate centrifuge speed. At present the centrifuge is run at approximately 900 revolutions per minute. This speed has been adequate in actual experiments with dogs where flow rates have been in the range of one to two liters per minute. Centrifuge speed is accurately indicated by a continuously running tachometer driven by the centrifuge. The latter is driven by a variable speed D.C. motor.

Application to animals. To date the complete respiratory function of 11 dogs has been replaced for 30 or more minutes by the machine and in one other dog for 12 minutes. Under pentobarbital anesthesia, the animals rebreathed nitrogen from a bag via an endotracheal tube with an inflated cuff. A tightly fitting face mask was additionally applied. The machine was primed with 2 liters of blood from another dog previously heparinized 5 mg per kg. The perfused animal also received this amount of heparin. Blood was withdrawn from the inferior vena cava via both femoral veins and returned via an external jugular vein. All animals except one survived the perfusions which were performed under nonsterile conditions, and the last 6 out of 9 survived in excellent health for more than one month.

Summary. 1. A heart-lung apparatus employing the principles of gas dispersion and centrifugation is described. 2. The apparatus presents certain advantages: a. No foreign substances other than anticoagulants need be added to the blood. b. Parts coming into contact with the blood can be sterilized by autoclaving. c. Over three liters of blood per minute can be completely oxygenated and defoamed in the apparatus in its present form, and this capacity can be greatly increased. 3. The apparatus can substitute for the respiratory function of the lungs in dogs for at least 30 minutes, with permanent survival of the animal.

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